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Testicular steroidogenesis and its regulation in the primate fetus and newborn

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Summary. The regulation of steroidogenesis of human fetal, rhesus monkey fetal and newborn testes was studied *in vitro* and *in vivo*. Human fetal testes (first half of gestation) bound specifically radioiodinated human chorionic gonadotropin (hCG) and responded with an increase of testosterone (T) production to physiologic levels (5 to 50 ng/ml) of hCG in incubations. Furthermore, placental effusate stimulated T release from fetal testicular minces in perifusion. In male monkeys chronically catheterized *in utero* (last third of gestation), intravenous administration of hCG or 100 μg/kg gonadotropin releasing hormone (GnRH) stimulated an increase in circulating T. Between two weeks and three months of postnatal age, this response clearly increased in both sensitivity and magnitude : doses as low as 10 to 20 μg/kg stimulated T responses 3-5 times higher than those seen *in utero*. Basal T levels also reached maximum concentrations during this period. Between three months and one year, the pituitary-testicular response to GnRH stimulation was gradually lost, and virtually no T response was seen by 1 year of age. Our observations confirmed the role of hCG as a tropic stimulus of human fetal testicular steroidogenesis during the first half of gestation. The monkey studies demonstrated that, during the second half of gestation, the pituitary-testicular axis was responsive to hypothalamic stimulation. This axis was less sensitive to GnRH *in utero* than immediately after birth. Postnatally, the response increased temporarily for 2-3 months, and was gradually lost thereafter. Accordingly, the loss of pituitary response to hypothalamic stimulation might be a major factor leading to low activity of prepubertal gonads.

Introduction.

Human testicular androgen production is activated for two short periods before puberty (see e. g. Faiman *et al.*, 1976 ; Forest *et al.*, 1976 ; Kaplan *et al.*, 1976). One of these active phases occurs in fetal life, during the first and second trimester of gestation, and the other is seen during the first months of postnatal life. The first phase of testicular activity is known to be of importance in the induction of male external genital differentiation (Jost *et al.*, 1973). No clear physiologic role has been yet assigned to the

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postnatal short active phase in testicular androgen production. Possible functions include masculinization of certain areas of the central nervous system, descent of the testes, timing of puberty and effects on sexual behavior.

The endocrine regulatory mechanisms involved in the temporarily high activity of fetal and newborn testes have not been elucidated. Indirect evidence, based upon simultaneous high levels of placental hCG and fetal testicular T in the first and second trimesters of gestation, suggests that hCG might be the tropic stimulus needed for fetal testicular activation. In addition, pharmacologic doses of hCG have been shown to stimulate T synthesis in incubation (Ahluwalia *et al.*, 1974) and tissue culture (Abramovich *et al.*, 1974). One aim of the present studies was to obtain direct information about the role of hCG in stimulation of human fetal testicular steroidogenesis.

Human fetal testicular steroidogenic activity remains low during the latter part of gestation. Some degree of activity, however, is present as shown by sex differences in amniotic fluid and cord blood T levels (Warne *et al.*, 1977 ; Forest and Cathiard, 1975). Nothing is known about the regulation of this testicular activity. Neither is it known by which mechanisms the temporarily high activity of testicular T production is attained after birth. Another aim of the present studies was to gain further insight into testicular regulation during the latter part of gestation as well as into the mechanisms of changes in gonadal regulation induced by birth. Rhesus monkeys were used as experimental animals in these studies. Since no measurable chorionic gonadotropin is present in the rhesus monkey circulation after the first third of gestation (Hodgen *et al.*, 1974, 1975), this animal is a very suitable model for studies of the development of fetal pituitary-gonadal interactions during the perinatal period.

Materials and methods.

Human fetal studies.

Tissue. — Early and mid-term human fetuses (8-18 weeks gestation) were obtained at the time of abortion for socio-medical reasons. The fetuses were delivered either by abdominal hysterotomy or by prostaglandin induction. The tissues were removed within 30 min. after delivery and the experiments were started immediately.

Assays of hCG *binding.* — Details of the procedure have been presented elsewhere (Huhtaniemi *et al.*, 1977*a*, 1978*a*). Highly purified NIH-hCG was radioiodinated with $Na^{125}I$ using the lactoperoxidase method. Fetal testes were homogenized in ice-cold buffer and aliquots of the homogenate were incubated in varying amounts of ^{125}I-hCG at 24 °C for 16 h. Non-specific binding was assessed in tubes containing an excess of cold hCG. After incubation, bound and unbound hormone were separated by centrifugation. The tubes were counted for ^{125}I-radioactivity and Scatchard-type plots were constructed to determine the binding characteristics of the tissue.

Incubation with hCG. — Aliquots of minced fetal testes were pre-incubated at 37 °C for 30 min. Thereafter, the buffer was changed to that containing 0, 0.5, 5 or 50 ng/ml of highly purified hCG (Huhtaniemi *et al.*, 1977*a*). Aliquots of the media were taken after 1, 2 and 3 hrs for T analysis (Korenbrot *et al.*, 1977). Media from one 2 hrs incubation were also analyzed for pregnenolone, progesterone,17-hydroxyprogesterone,

dehydroepiandrosterone, 5α-dihydrotestosterone and androstenedione, essentially as described by Jänne *et al.* (1974) and Hammond *et al.* (1977).

Perifusions. — The perifusion apparatus is described in detail elsewhere (Huhtaniemi and Lautala, 1979). Minced fetal testes were perifused with the buffer alone for the first 60-90 min. of the experiment. Thereafter, another perifusion chamber, containing a 0.5 g piece of placenta, was connected to the buffer flow. The buffer was first introduced to the placental chamber and from there to the chamber containing the testes. The perifusion was continued for 1.5 to 2.5 hrs and the buffer was collected in 5 min. fractions. The fractions were analyzed for T, progesterone and hCG.

Monkey experiments.

Fetal testis hCG binding assays and incubations with hCG were performed essentially as described above for human fetuses. Fetuses (125-145 days gestation) were obtained either following premature delivery or Cesarean section. The mean length of gestation in this colony is 168 days.

In vivo experiments. — Details of the surgical techniques used for chronic catheterization of rhesus monkey fetuses *in utero* have been described elsewhere (Jaffe *et al.*, 1977 ; Huhtaniemi *et al.*, 1977*b*). The catheterization was performed during the last third of gestation. These fetal monkeys were challenged *in utero* with intravenous bolus injections of 10 or 100 IU of hCG or 10-50 μg (= 20-100 μg/kg) of GnRH. Blood samples were taken before the injections and up to 2 hrs after them.

Newborn monkeys of 1-4 days of age were catheterized via the carotid artery or jugular vein, and, similar to the fetuses, the newborn male monkeys received bolus injections of 10-100 μg/kg of GnRH. Blood samples were collected in the same manner as from the fetuses. Older animals were first infused via peripheral veins and blood was obtained from femoral vessels.

A slow-release gel preparation of GnRH (500 μg) in polyvinyl pyrrolidone was administered subcutaneously bimonthly to three male monkeys throughout the first year of life. The vehicle alone was administered in an identical fashion to two animals. Blood samples from these animals were collected prior to each GnRH injection.

T was measured in all plasma samples collected, as described above. Several plasma samples were also measured for LH activity, using a rat Leydig cell *in vitro* bioassay (Huhtaniemi *et al.*, 1979), modified from that described by Moyle and Ramachandran (1973).

Results.

Human fetal studies.

The ^{125}I-hCG binding capacity of five human fetal testes was 32.5 ± 7.1 (SD) ng/g wet tissue and the equilibrium association constant 1.07 ± 0.12 × 10^{10} M^{-1} (Huhtaniemi *et al.*, 1977*a*). The crownrump length of the fetuses varied from 13.5 to 19.5 cm. No clear correlation of binding to fetal size could be seen in this small number of individual samples.

Figure 1 Illustrates results of a typical incubation, in which T synthesis of fetal testicular minces was stimulated with increasing doses of hCG. Incubation with six individual testes demonstrated that maximal T release into the incubation medium was attained in hCG concentrations ranging from 5 to 50 ng/ml (Huhtaniemi *et al.*, 1977*a*).

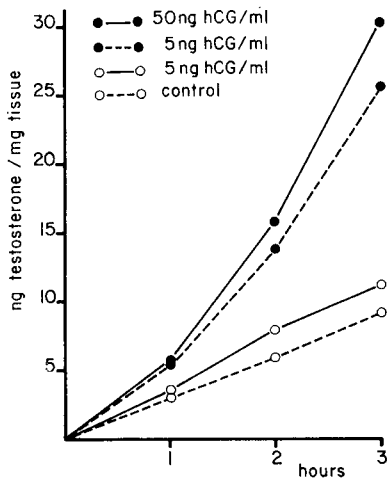

FIG. 1.

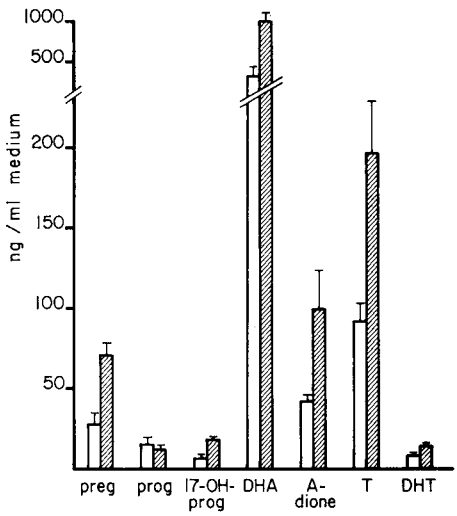

FIG. 2.

FIG. 1. — *Effect of different concentrations of* hCG *on endogenous T production of minced human fetal testis in a typical incubation* (fetal gestational age 16 weeks). Each point represents the mean of duplicate incubations (Huhtaniemi *et al.*, 1977*a*).

FIG. 2. — *Formation of pregnenolone* (preg.), *progesterone* (prog.), *17-hydroxyprogesterone* (17-OH-prog.), *dehydroepiandrosterone* (DHA), *androstenedione* (A-dione), *testosterone* (T) *and dihydrotestosterone* (DHT) *from endogenous precursors in fetal testicular minces.* Steroids were measured in the incubation medium after a 2-h incubation. The open bars indicate the controls and the shaded bars indicate samples incubated in the presence of 50 ng/ml of hCG. The mean ± S. D. of quadruplicate incubations is given. The fetal age was 14 weeks of gestation (Huhtaniemi and Lautala, 1979).

In one experiment, minces in quadruplicate were incubated in the presence and absence of 50 ng/ml hCG and the 2h media were analyzed for several other steroids (fig. 2) (Huhtaniemi and Lautala, 1979). It was seen that, in addition to T, the formation of pregnenolone, 17-hydroxyprogesterone, dehydroepiandrosterone, androstenedione and 5α-dihydrotestoterone was stimulated during the incubation. The level of progesterone, however, was not clearly changed.

The results of one representative perifusion experiment are shown in figure 3. When the testes were perfused alone, typically, the first 10-15 fractions contained high levels of T, before a steady-state in its release was attained. During the latter period, the other perifusion chamber, containing the piece of placenta, was connected above that containing the testes. An immediate increase in the concentrations of the two placental hormones, progesterone and hCG, was seen in the effusate. Thereafter,

a clear increase in T level of the perifusate was seen. In the four experiments performed, the increase in T levels was 2-3-fold in each case (Huhtaniemi and Lautala, 1979).

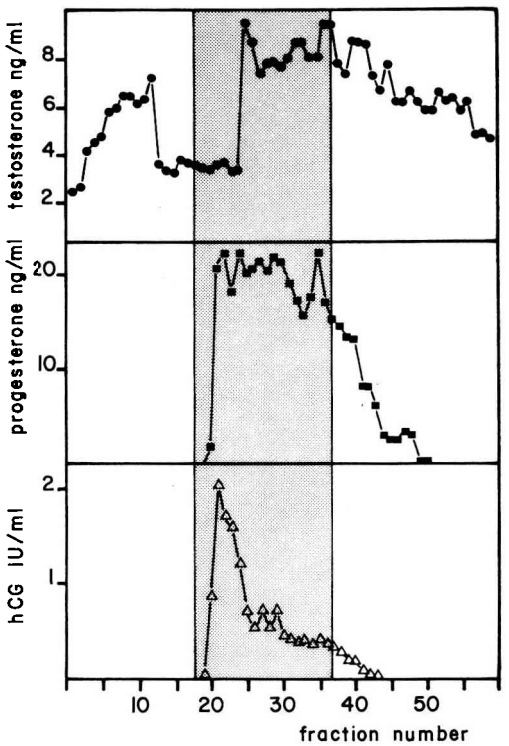

FIG. 3. — *Testosterone, progesterone and* hCG *concentrations in the perifusion media of a typical experiment.* The shaded area indicates the time when the placental chamber was connected to the system. The buffer fractions were collected in 5 min. aliquots. The age of the fetus used in this experiment was 14 weeks (Huhtaniemi and Lautala, 1979).

Monkey fetal studies.

Specific binding of hCG also was demonstrated in monkey fetal testes. In one of three experiments, the amount of tissue was sufficient to permit a Scatchard analysis (Huhtaniemi *et al.*, 1977*b*) and the binding capacity was 102 ng/g wet tissue and the equilibrium association constant was $1.87 \times 10^{10}\ M^{-1}$. *In vitro* stimulation of minced testis steroidogenesis by hCG yielded very similar results to those obtained with human fetal testes : hCG levels of 5 ng/ml or above stimulated maximal T release into the incubation media (results not shown).

The basal T level in male monkeys chronically catheterized *in utero* was 1.08 ± 0.14 (SD, $n = 7$) ng/ml (fig. 4). Administration of 10 or 100 IU hCG into the fetal circulation stimulated a clear increase in circulating T levels (fig. 5). Other fetuses were challenged *in utero* with bolus injections of 10 or 50 μg GnRH (fig. 6). The lower dose induced a

clear increase in T in only one of 4 fetuses, while the higher 50 μg dose significantly increased plasma T in all three fetuses studied. The mean maximal response was about 100 p. 100.

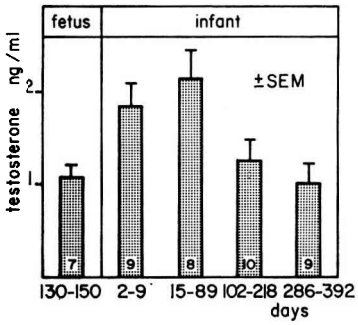

FIG. 4.

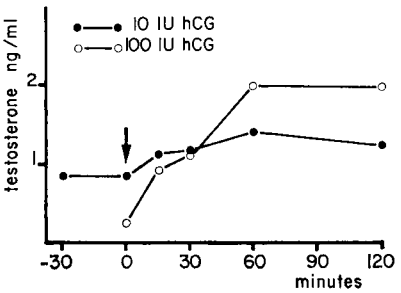

FIG. 5.

FIG. 4. — *Mean basal levels* (± SEM) *of circulating* T *in male fetal and infant monkeys grouped according to age.* The number of individuals in each group is indicated by the number superimposed on the bar (Huhtaniemi *et al.*, 1979).

FIG. 5. — *Effect of intravenous infusions of* 10 *and* 100 IU *of* hCG *on mean fetal serum* T *level in utero.* Two chronically catheterized monkeys (o, •) were injected at the time indicated by the arrow (Huhtaniemi *et al.*, 1977*b*).

Newborn monkey studies.

Basal T level in 2-9 days old newborns was 1.80 ± 0.21 (SD, n = 9) ng/ml (fig. 4), reached a maximum between days 15-89 (2.14 ± 0.29 ng/ml, SD, n = 8) and gradually

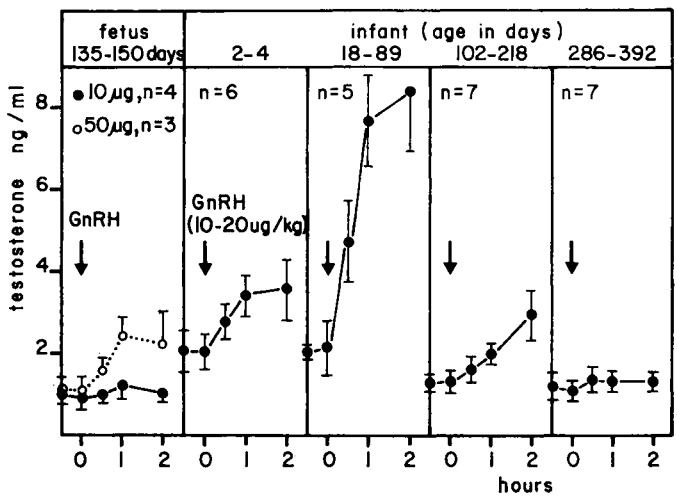

FIG. 6. — *Mean response of circulating* T *to* GnRH *infusions in male fetal and infant monkeys.* Bolus intravenous infusions of GnRH (10 μg, ––•––, or 50 μg, ––o––, in fetuses ; 10-20 μg/kg in infants) were administered at the times designated by the arrows. The number of different individuals in each group is indicated by « n » (Huhtaniemi *et al.*, 1979).

decreased thereafter, being 1.25 ± 0.17 (SD, n = 10) ng/ml on days 102-218 (Huhtaniemi *et al.*, 1979). When male monkeys 2-4 days of age were infused with as little as 3-10 μg GnRH (10-20 μg/kg), a clear response was seen in 5 out of 6 monkeys (fig. 6). The mean maximal response was 105 p. 100. In older animals, 18-89 days, the same dose of GnRH elicited a 3-5-fold response, compared to the fetuses and younger newborns. The mean response in the 5 subjects of this group was 367 p. 100. After about 3 months of age, the T response to GnRH stimulation gradually decreased. In the 102-218 days group, the mean T response was 133 p. 100 of the basal level and in the oldest, 286-392 days group, only one out of 7 animals showed a clear T response to GnRH injection.

Figure 7 illustrates preliminary results of concomitant changes in the LH levels in response to the GnRH injections. One experiment from each age group is depicted. It can be seen that the increases of LH and T after hypothalamic stimulation correlate very well.

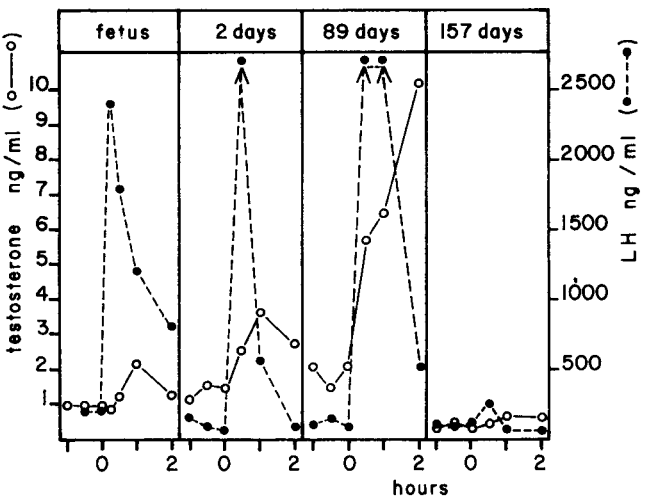

FIG. 7. — *Effect of* GnRH *on male fetal and infant plasma* T (— o —) *and* LH (— • —). GnRH infusions (5-50 μg) occurred at 0 hr.

Data obtained from the experiment with subcutaneous injections of 500 μg GnRH in slow-release form are seen in figure 8. High levels of T (between 5 and 10 ng/ml) were seen in the three GnRH-treated animals up to about 70 days of age. The concomitant T levels in the two control animals stayed (with one exception of 3.5 ng/ml) below the level of 3 ng/ml.After about 80 days of age, until 360 days, no clear difference was seen between the controls and GnRH treated animals, despite the bimonthly 500 μg GnRH injonctions.

Discussion.

Our observations demonstrate that human fetal testes can bind specifically hCG at the time of active androgen production in the first and second trimester of gestation.

The association constant of binding ($1.07 \pm 0.12 \times 10^{10}\ M^{-1}$) was very similar to those demonstrated for testes of rats and monkeys (Catt *et al.*, 1973 ; Huhtaniemi *et al.*,1977*b*), and also to adult human testes (Huhtaniemi *et al.*, unpublished observations). The binding capacity of the tissue ranged from 25.6 to 42.2 ng/g which also is very similar to that of adult human testes (Huhtaniemi *et al.*, 1978*b*).

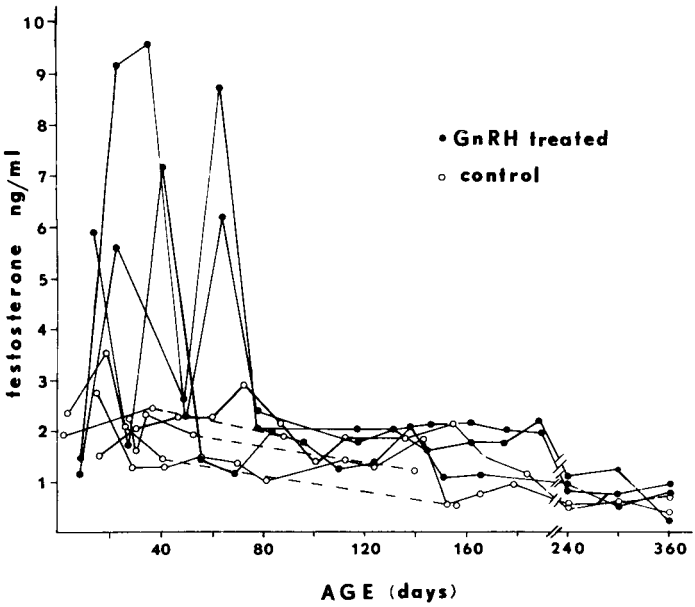

FIG. 8. — GnRH *effects on the response of plasma* T *in individual infant monkeys.* A « slow-release » form of GnRH (500 μg, closed circles), or saline in the same vehicle (open circles) was injected subcutaneously once every 2 weeks. The T levels shown are just prior to the biweekly injection (Huhtaniemi *et al.*, 1979).

Both the incubation and perifusion studies demonstrated that hCG, in physiologic concentrations, was able to stimulate fetal testicular steroidogenesis. Doses between 5 and 50 ng/ml were effective, and the fetal serum hCG-concentration at this time of gestation is about 35 ng/ml (Clements *et al.*, 1976). Whether placental hCG is solely responsible for the stimulation remains unclear. The contribution of fetal pituitary gonadotropins, however, seems unlikely, since these substances do not appear in the fetal circulation in appreciable amounts until about 18-20 weeks of gestation (Kaplan *et al.*, 1976). Stimulation of fetal testes by luteinizing hormone and/or chorionic gonadotropin also has been demonstrated in fetal rat (Warren *et al.*, 1975 ; Picon and Ktorza, 1976) and fetal rabbit testes (George *et al.*, 1978).

Testicular T may either be formed *de novo* or from endogenous steroid precursors such as placental progesterone. Since, however, no exogenous substrates were used in the incubations, and since, after 3 hrs, the hCG-stimulated testes had synthesized, on an average, 5 times more T than could be accounted for by the concentrations of endogenous C_{19} and C_{21} steroids in the tissue (Huhtaniemi *et al.*, 1970), *de novo* formation or synthesis from sterol precursors seems more likely than formation from

placental progesterone. *De novo* biosynthesis of T from acetate has been demonstrated previously in human fetal testes *in vitro* (Serra *et al.*, 1970). Analysis of a number of other endogenous steroids in the medium further emphasizes the *de novo* formation of T, since the concentrations of several of its precursors also increased during the incubation.

The monkey fetal studies demonstrate that, at least in this primate, testicular steroidogenesis is functional near term. The testis binds hCG specifically and responds with increased T output to this tropic stimulation. Because no chorionic gonadotropin is present in the rhesus monkey circulation after the first third of gestation (Hodgen *et al.*, 1975), the origin of the gonadotropic stimulation *in vivo* is of interest. Since GnRH was able to increase circulating T in male fetuses, the role of a functional pituitary-gonadal axis seems likely. We found that the pituitary gonadal axis also was functional after birth and that it displayed an increased response to GnRH until about 3 months of age, and gradually disappeared thereafter and was virtually unresponsive by the end of the first year of life.

An increase in the testicular T response to GnRH was very clear after birth. In the fetus, only a 100 μg/kg intravenous dose of GnRH was effective consistently, while from the second to fourth day after birth, doses as low as 10-20 μg/kg elicited clear positive responses. Besides the sensitivity, the magnitude of the T response was amplified after birth. The maximal T response to GnRH *in utero* was about 100 p. 100, while 14-89 days after birth the mean response averaged 380 p. 100. The magnitude of response decreased after about 3 months, so that the mean maximal response in infants 3-7 months of age was similar in magnitude to that seen immediately after birth and *in utero*. Later on, the response to this dose range of GnRH was negligible in most monkeys 9-13 months of age. Our preliminary LH determinations show that the changes in LH response to GnRH parallel those observed in T.

Changes in circulating steroid and/or protein hormones before and after birth may explain the observed changes in responsiveness to GnRH. There is evidence that estrogens exert an inhibitory effect on gonadotropin release in adult males (Verjans *et al.*, 1974), as well as on LH effects on the testis (Grumbach *et al.*, 1974 ; Huhtaniemi *et al.*, 1978*b*). Thus, the elimination of large amounts of placental estrogens from the fetal circulation after birth may explain the observed increase in testicular androgen production at this time. The possible inhibiting action of increased levels of prolactin on the stimulation of steroidogenesis by fetal LH has been discussed by Kaplan and Grumbach (1976). Effects of other placental steroids (e. *g.* progesterone) and protein hormones on the function of the fetal hypothalamic-pituitary-gonadal circuit have not been explored. Furthermore, the factors that may be involved in the decrease in T responsiveness seen after three months, and whether there is a developmental change in the hypothalamus or pituitary, regulated by these factors, remain to be elucidated.

In humans, a low level of circulating endogenous GnRH has been suggested as a reason for the diminished LH release in response to exogenous GnRH in prepubertal children (Grumbach *et al.*, 1974). This may not be the only reason for low responsivity of the prepubertal pituitary to hypothalamic stimulation, since, in the present experiments, the response of the monkey fetal pituitary-gonadal axis to continual administration fo GnRH in slow-release form was not seen to extend the sensitive period beyond 3 months of age. Thus, continual treatment with high doses of exoge-

nous GnRH was not sufficient to maintain the sensitivity of the pituitary-gonadal axis at the high neonatal level. It also seems unlikely that the production of high levels of T decreases the responsiveness of the pituitary-gonadal axis, since the repeated exposure to GnRH from 2 weeks of age, resulting in T levels higher than those in the controls, did not result in decreased responsiveness to GnRH earlier than 3 months of age.

Our findings agree well with observations on human male infants (Faiman *et al.*, 1976 ; Forest *et al.*, 1976). As in the monkey, there is a peak of circulating T in human male infants 1-3 months old. This peak is simultaneous with an increase in gonadotropins in the infant circulation. There also is some evidence that the human newborn pituitary is sensitive to GnRH just after birth, while the prepubertal pituitary is fairly unresponsive (Suwa *et al.*, 1974 ; Betend *et al.*, 1975 ; Delitala *et al.*, 1978). These similarities would suggest that the rhesus monkey is a useful experimental model for more detailed studies on the perinatal development of hypothalamic-pituitary-gonadal interactions.

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Résumé. La régulation de la stéroïdogenèse par le testicule fœtal ou postnatal de l'homme et du macaque rhésus a été étudiée *in vivo* et *in vitro*. Le testicule fœtal humain pendant la première partie de la gestation fixe spécifiquement l'hormone gonadotrope chorionique humaine marquée et répond par une sécrétion accrue de testostérone à des niveaux physiologiques de HCG (5-50 ng/ml) au cours des incubations. De plus, le liquide de perfusion du placenta stimule la libération de testostérone à partir de coupes fines de testicule fœtal en périfusion. L'administration d'HCG ou de GnRH (100 μg/kg) à des fœtus mâles de rhésus avec cathéter permanent (dernier tiers de la gestation) entraîne une augmentation de la testostérone plasmatique. Entre deux et trois semaines après la naissance la réponse augmente en amplitude et en sensibilité. Des doses aussi faibles que 10 à 20 μg/kg de GnRH stimulent 3 à 5 fois plus la testostéronémie qu'*in utero*. Le taux de base de la testostérone atteint son maximum à ce moment. Entre 3 mois et un an la sensibilité hypophysaire au GnRH disparaît progressivement et pratiquement aucune réponse au niveau de la testostérone plasmatique n'est observable à l'âge de 1 an.

Nos observations conduisent à penser que HCG stimule la stéroïdogenèse du testicule fœtal humain pendant la première partie de la gestation. L'étude sur le macaque montre que pendant la seconde partie de la gestation l'axe hypophyse-testicule est sensible à la stimulation hypothalamique. Cette sensibilité est moindre *in utero* qu'immédiatement après la naissance. La réponse augmente alors pendant 2 à 3 mois et ensuite s'efface graduellement. Ainsi la perte de sensibilité de l'hypophyse au facteur de stimulation hypothalamique pourrait être un des facteurs essentiels entraînant la faible activité des gonades avant que ne débute la puberté.

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