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REGULATION OF THE PRIMATE FETAL ADRENAL GLAND AND TESTIS *IN VITRO* AND *IN VIVO*

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SUMMARY

In vitro experiments with the separated fetal zone (FZ) and definitive zone (DZ) of the human fetal adrenal (10–20 weeks) demonstrated ACTH stimulation of cortisol and binding in the DZ. Dehydroepiandrosterone sulfate (DHAS), produced primarily in the FZ, was stimulated inconsistently by ACTH, but consistently by hCG. Thus, the DZ appears regulated by ACTH, and the FZ principally by hCG in the first half of gestation.

To assess adrenal regulation in the primate during the second half of gestation, under more physiologic circumstances, studies were carried out in a chronic fetal rhesus monkey preparation *in utero* in which fetuses (130–145 d gestation) were catheterized, replaced in the uterus, and the pregnancy allowed to continue up to 14 days. Dexamethasone administered to the fetus suppressed DHAS, cortisol and ACTH. Seven fetuses were challenged with ACTH. An increase in cortisol production was only observed in two; no significant stimulation was seen in the others. The challenge with ACTH was given to two infant monkeys on the first day of life. Cortisol levels increased 10-fold after ACTH stimulation. The data suggest some intrauterine factor which blocks the response to ACTH.

In studies of fetal monkey testicular regulation, *in vitro* and *in vivo*, specific hCG binding was demonstrated in testicular homogenates and hCG stimulated testosterone (T) production in testicular minces. *In utero* intra-arterial administration of hCG to fetuses resulted in a 4-fold increase in fetal serum T. Other fetuses were challenged with bolus infusions of 10 and 50 μg gonadotropin releasing hormone (GnRH). Only the higher dose resulted in fetal T stimulation. In newborn monkeys, the lower 10 μg dose of GnRH also caused an increase in serum T. Thus, the pituitary-adrenal and pituitary-gonadal axes appear functional in the third trimester in this species, and target gland sensitivity appears to increase after birth.

INTRODUCTION

The suggested role of the fetal adrenal gland in the maturation of the fetal lung and its possible role in the initiation of labor in certain species, as well as the known role of cortisol in enzyme induction and fetal development have furnished the impetus for studies, both *in vitro* and *in vivo* concerning the regulation and function of the primate fetal adrenal gland. In a like manner, the importance of fetal androgens in male sexual differentiation has prompted an exploration of the regulation and function of the primate fetal testis.

Three groups of studies will be described in the experiments to be presented: (1) *in vitro* studies of the human fetal adrenal gland; (2) *in vivo* studies of the monkey fetal adrenal gland; and (3) *in vitro* and *in vivo* studies of the monkey fetal testis.

I. *IN VITRO* STUDIES IN THE HUMAN FETAL ADRENAL GLAND

A striking and unique aspect of human intrauterine fetal life is the development of the adrenal glands. During early fetal life, the adrenals grow rapidly and, by the end of the first trimester, have attained a size equal to or larger than that of the fetal kidneys. At term, on a proportionate weight basis, they are 10–20 times larger than the adult adrenals. The bulk of the fetal adrenal mass is attributable to a central, 'fetal zone' which comprises about 80% of the vol. of the gland. Coincident with delivery, there is a rapid and extensive degenerative process leading to the loss of the fetal zone.

The human fetal adrenal gland is an active androgen secreting organ. It provides most of the dehydroepiandrosterone sulfate (DHAS) utilized as a precursor of placental estrogens [1–3]. Further, incubations with labelled acetate [4] and progesterone [5] and *in vivo* perfusions with radio-labelled progesterone [2], have demonstrated that it also has the capacity to synthesize cortisol as early as 13 weeks of gestation. Judging from histologic appearance, the major steroidogenic activity appears to be localized in the cells of the fetal zone [6].

The marked diminution or absence of the fetal zone in many anencephalics [7], as well as the observation that steroid (DHAS and estrogen) concentrations are low in these pregnancies and in mothers treated with synthetic glucocorticoids [8], suggests that steroid

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production by the fetal adrenal gland is controlled by hormones from the pituitary, possibly ACTH.

The investigations to be described were designed to evaluate the relative contribution to cortisol and DHAS production by the fetal and definitive zones of the human fetal adrenal gland, and the possible regulatory roles of fetal pituitary and placental tropic hormones upon the secretion of these steroids. Toward this end, corollary studies of regulation and function have been performed by assessing steroid production in a superfusion system and studying binding of tropic hormones in glands at similar stages of pregnancy.

MATERIALS AND METHODS

A. Steroid production by the definitive and fetal zones

Adrenal and pituitary glands, from 15 fetuses 10–21 weeks gestation, were obtained in accordance with recently promulgated suggested federal guidelines. Most of the fetal tissue was obtained following prostaglandin-induced pregnancy termination. Fetal pituitary glands were frozen and later thawed to prepare a pituitary homogenate.

To study secretion, the separated zones (fetal and definitive) of the fetal adrenal were finely minced, and both zones were separately and simultaneously superfused with Krebs–Ringer Bicarbonate Glucose Buffer (KRBG, pH 7.4). The apparatus used in the superfusion was similar to that described by Gurpide and Welch[9]. The buffer was continuously gassed with 95% O_2–5% CO_2. The eluate was collected constantly in five minute fractions (2 ml/5 min), for five or six h. The usual protocol was to superfuse the tissue with buffer alone for 90–120 min then add hormone to the medium and continue the experiments for another 2–2.5 h.

Cortisol was measured by radioimmunoassay. Total androgen secretion was quantitated utilizing the technique described by Walsh *et al.*[10], which measures tritiated water formed during aromatization by a placental microsome preparation. Results are expressed in terms of 'DHAS' as this is the major androgen secreted by the human fetal adrenal gland [11]. Other aromatizable androgens also would be measured by this assay.

B. Effect of hCG on androgen secretion by the isolated fetal zone of the human fetal adrenal gland

Adrenal glands were dissected to separate the fetal and definitive zones, and fetal zone minces were superfused as described above. For each individual gland, control and experimental (250 ng/ml hCG added to the buffer) superfusions were performed simultaneously. Total aromatizable androgen, expressed as 'DHAS', was assayed as described above.

C. ACTH binding to the human fetal adrenal gland

Fetal adrenal glands from fetuses 10–20 weeks gestational age were dissected to separate the definitive and fetal zones, and each zone was finely minced. Synthetic ACTH (1–24), was iodinated and purified by glass adsorption (specific activity 500 μCi/mg). Minces of the isolated zones of the fetal adrenal gland were incubated in KRBG with [^{125}I]-ACTH in the presence or absence of 500 ng unlabelled ACTH. After incubation, the tissue was centrifuged, and the tissue pellet transferred to a clean tube. Radioactivity remaining in the tissue was counted in a gamma counter. The results were expressed as:

$$\frac{\%\text{ total radioactivity displaced by excess unlabelled ACTH (specific binding)}}{\text{total binding}}$$

RESULTS

A. Steroid production by the definitive and fetal zones

1. *Cortisol secretion by the isolated definitive zone.* (a) *Response to 250 ng/ml ACTH.* During the superfusion, cortisol secretion is initially high (5–15 ng/100 mg tissue/ml), then drops within 1 h to between 2.5 and 6 ng/100 mg/ml. Addition of ACTH, 250 ng/ml to the superfusion medium produces a 2–5 fold increase over baseline values, raising cortisol secretion from 50–150% over the values seen at the beginning of the experiment. Stimulation of cortisol secretion is maintained for variable periods, following which secretion tends to diminish in spite of continuous ACTH infusion.

(b) *Response to lower doses of ACTH.* Although 250 ng/ml of ACTH produces a consistent increase in cortisol production, ACTH levels in the fetal circulation are of the order of 250 pg/ml [12]. To study the effect of lower amounts of ACTH on cortisol production by the definitive zone, two glands were stimulated consecutively with doses of ACTH of 0.25 and 2.5 ng/ml, two with 2.5 ng and 25 ng/ml, and one gland was stimulated with 2.5, 25 and 250 ng/ml. None of the glands responded to 0.25 ng/ml ACTH. Only one of the glands (a hysterotomy specimen) responded to 2.5 ng/ml ACTH, and a slight but significant response to 25 ng/ml was seen in two of three glands. A clear response, proportional to the increasing doses of ACTH, was seen in the gland exposed to the 2.5, 25 and 250 ng/ml doses (Fig. 1).

(c) *Response to fetal pituitary homogenate.* The isolated fetal and definitive zones of three glands were superfused with a fetal pituitary homogenate. Cortisol secretion by the definitive zone increased after stimulation with the homogenate in all the glands, although a large variation was seen in the response.

2. *Cortisol secretion by the isolated fetal zone.* Initial cortisol secretion by the isolated fetal zones, ranging between 1 and 6 ng/100 mg/ml, was lower than the output of the definitive zones from the same glands described in Section 1(a). Secretion of cortisol decreased throughout the experiment, or was maintained at a low level, and there was no stimulation by the addition of ACTH or pituitary homogenate.

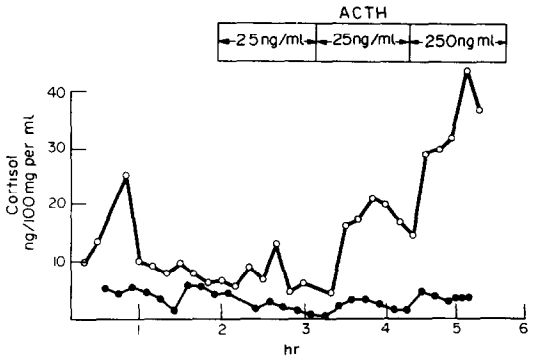

Fig. 1. Cortisol secretion by minces of isolated definitive (open circles) and fetal (closed circles) zone of the human fetal adrenal gland in response to treatment with increasing doses of ACTH. Tissue was superfused with KRBG for 2 h, then exposed successively to buffer containing 2.5, 25 and 250 ng/ml ACTH. From Serón-Ferré, M., Lawrence, C. C., and Jaffe, R. B. (Submitted for publication).

3. *'DHAS' secretion by the isolated definitive zone.* 'DHAS' secretion by the isolated definitive zone was much lower than that by the fetal zone (10–30 ng/100 mg/ml *vs* 100 ng/100 mg/ml respectively) and was not stimulated by ACTH or pituitary homogenate in any of the experiments.

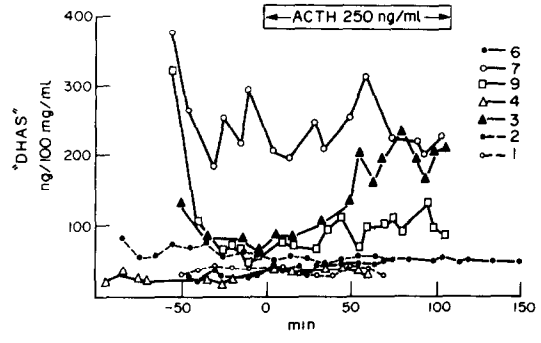

Fig. 2a. Dehydroepiandrosterone sulfate ('DHAS') secretion by minces of fetal zone of the human fetal adrenal gland. Glands were superfused with KRBG buffer for 90 or 120 min before addition of ACTH to the superfusate. From Serón-Ferré, M., Lawrence, C. C., and Jaffe, R. B. (Submitted for publication).

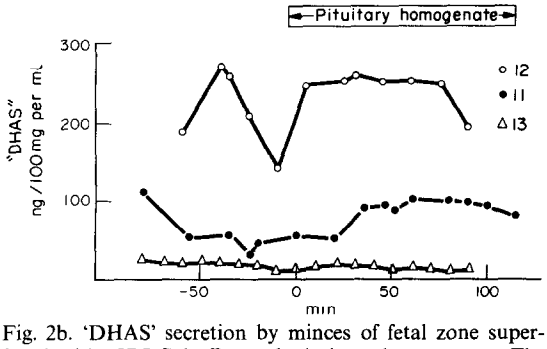

Fig. 2b. 'DHAS' secretion by minces of fetal zone superfused with KRBG buffer and pituitary homogenate. The pituitary homogenate was added to the system after presuperfusion with KRBG for 100 min. From Serón-Ferré, M., Lawrence, C. C., and Jaffe, R. B. (Submitted for publication).

4. *'DHAS' secretion by the isolated fetal zone.* (a) *Response to ACTH.* Figure 2a shows the composite graph of 'DHAS' secretion by the fetal zones of five adrenal glands stimulated with 250 ng/ml ACTH. Two control glands also are illustrated. Initial secretion was between 100 and 300 ng/100 mg/ml tissue, and diminished after one hr to levels under 100 ng/100 mg/ml. Stimulation with 250 ng/ml ACTH increased 'DHAS' production in three of these five glands. Treatment of five other glands with lower doses of ACTH (0.25 ng/ml, 2.5 ng/ml and 25 ng/ml) showed a response to the 25 ng/ml dose in three.

(b) *Response to pituitary homogenate.* 'DHAS' secretion by the fetal zones of the three glands superfused with pituitary homogenate is seen in Fig. 2b. An increase in 'DHAS' was seen in the fetal zone of the same two glands that showed a clear cortisol response in the definitive zone.

B. Effect of hCG on androgen secretion by the isolated fetal zone

Data concerning 'DHAS' production after superfusion of the isolated fetal zone, in the presence and absence of added hCG, are presented in Fig. 3. In all cases, initial 'DHAS' concentrations were high and decreased during the first h of superfusion, regardless of the presence or absence of hCG in the medium. After this time, values stabilized in the control experiments and started increasing in the presence of hCG. Figure 3 illustrates the mean 'DHAS' values of five experiments. No significant difference was seen between hCG and control superfusions during the first h. 'DHAS' secretion in the superfusions in which hCG was added rose significantly over the control by 90 min, and reached maximal values by 2 h. These increased values remained constant during the rest of the superfusion.

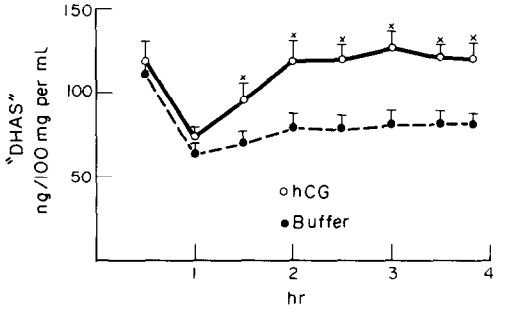

Fig. 3. 'DHAS' levels during superfusion of isolated fetal zone of the human fetal adrenal gland in the presence and absence of hCG. Each point represents mean and standard error of the average 'DHAS' concentration (ng/100 mg/ml) in samples of effluent collected every 10 min during that time interval. $P < 0.005$. From Serón-Ferré, M., Lawrence, C. C. and Jaffe, R. B. (Submitted for publication).

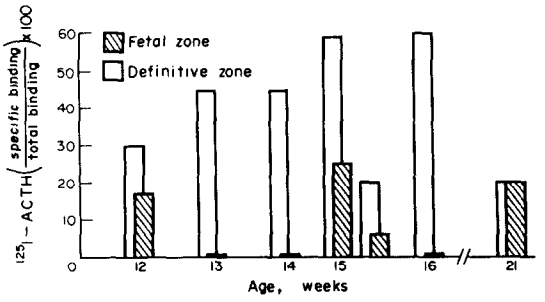

Fig. 4. [^{125}I]-ACTH binding to minces of separated definitive and fetal zones of the human fetal adrenal gland at different gestational ages.

C. *ACTH binding*

Figure 4 illustrates the binding of ACTH to the separated definitive and fetal zone minces of seven adrenal glands obtained from fetuses of different gestational ages. ACTH binding in the definitive zone was observed in all cases. ACTH binding to the fetal zone was found in four of the seven glands studied. In six of the glands, more radioactivity was found to be bound to the definitive zone than to the fetal zone of the same gland. In the seventh, the oldest fetus, binding was approximately the same in each zone.

DISCUSSION

The data indicate that there is a functional specialization of the fetal adrenal in regard to steroid production and its regulation. The isolated definitive (adult) zone of the gland has the capacity to form cortisol when stimulated with ACTH either *de novo* or from endogenous precursors, as exogenous precursors were not added in our experiments. Androgen production by this zone was negligible, and was not stimulated by ACTH. In contrast, androgens were the major secretory products of the isolated fetal zone of the adrenal. Relatively little cortisol was secreted by this zone of the gland.

The capacity for cortisol production by homogenates or minces of the whole fetal adrenal gland has been demonstrated by several investigators utilizing radioactive precursors [2, 5]. Fetal adrenal cell cultures, composed primarily of definitive zone cells, also secrete cortisol, and this secretion is stimulated by ACTH [13]. Further, adrenal glands from anencephalic fetuses, composed mainly of definitive zone cells, have been shown to be capable of making cortisol from exogenous radioactive precursors [14]. In our experiments, the definitive zone of the fetal adrenal gland responded to ACTH with increased cortisol secretion as early as ten weeks of gestation. The response seemed to be of similar magnitude (approximately a five-fold increase) at the various gestational ages studied, through 20 weeks. The magnitude of the cortisol response to ACTH, while not apparently related to fetal age, did seem to reflect the 'viability' of the tissue. The pattern of the response to ACTH is similar to the response of the adult rat adrenal in a comparable superfusion system [15].

The cortisol response to ACTH was seen to be dose-related. The requirement of supraphysiologic doses of ACTH to stimulate cortisol production by the definitive zone may just be an artifact of the *in vitro* system as contrasted with studies *in vivo*. Decreased sensitivity to ACTH *in vitro* also has been observed in superfusions of rat adrenal glands [16].

In the superfusion system, the isolated fetal zones secrete androgens and very little cortisol, in agreement with the reported lack of 3β-hydroxysteroid dehydrogenase activity in this zone [16]. Androgen (DHAS) has been found to be the major secretory product of this zone following incubation with radioactive precursors [4]. Further, quantitation of endogenous free and conjugated steroids in the whole fetal adrenal revealed DHAS to be the major androgen present [17] and the steroid produced in major quantities during incubation [1]. In our experiments, hCG rather than ACTH consistently stimulated DHAS production. Effects of hCG on fetal adrenal morphology [6], incorporation of radioactive acetate into cholesterol [18] and stimulation of DHAS in the newborn [19] have been reported. A tropic role of hCG for the human fetal adrenal gland is consistent with the finding that this gland is normal early in pregnancy in the absence of a functional hypothalamic-pituitary axis [7]. In the binding studies, specific binding of iodinated ACTH was consistently found in the definitive zone, while binding in the fetal zone was seen in only four of the seven cases. Our *in vitro* superfusions demonstrated that indeed the definitive zone responds to ACTH and that the fetal zone responds inconsistently to ACTH at mid-gestation. ACTH may not be the sole regulatory factor for the fetal zone before 20 weeks of gestation as the adrenal is normal in anencephalic fetuses at this time [7], and as we have demonstrated hCG capable of regulating fetal zone steroidogenesis *in vitro* at this gestational age. Later in gestation, however, the fetal zone of the fetal adrenal gland probably develops responsiveness to ACTH. If this is the case, the variable binding of ACTH by the fetal zone observed in these experiments may indicate a period during which the capacity to respond to ACTH, as reflected by the appearance of binding sites for this hormone, is just beginning to occur.

We propose that, while ACTH serves as the regulatory hormone for the definitive zone throughout gestation, hCG is a regulating hormone for the fetal zone early in gestation in a fashion similar to that proposed for the fetal testis, and that ACTH and/or other fetal pituitary hormones become more important as gestation progresses.

II. *IN VIVO* STUDIES OF THE FETAL MONKEY ADRENAL GLAND

In vitro and acute *in vivo* studies have established that the primate fetal adrenal gland produces both androgens (such as DHAS) and cortisol. Our studies presented above have shown that the human fetal adrenal gland can respond to certain trophic hormones. While such studies have yielded much information about these steroid biosynthetic pathways and their regulation in the *in vitro* situation, the validity of these observations under *in vivo* conditions remains to be determined. To integrate the *in vitro* data with the situation obtaining *in vivo*, we have adapted a preparation developed by Martin *et al.*[20], utilizing the chronically catheterized fetal rhesus monkey *in utero*, that allows us to pose questions concerning the regulation of the primate fetal adrenal in a more physiologic manner.

Using this model, we have studied the role of ACTH in the regulation of fetal DHAS and cortisol secretion by studying (1) basal levels of cortisol in the fetal circulation *in utero*, (2) changes in circulating cortisol in response to ACTH challenges, (3) fetal ACTH and cortisol responses to treatment with dexamethasone and (4) the response of the fetus and neonate to a challenge with ACTH.

MATERIALS AND METHODS

Monkeys with known dates of conception were studied at 129–145 days of pregnancy. They were pretreated for 3 days prior to surgery with 50 mg progesterone. A peripheral vein was catheterized and an intravenous infusion of 0.9% NaCl and fenoprofen (0.2 mg/min) was begun.

The uterus was exposed through a vertical midline incision. After identification of the placenta by palpation, a transverse incision was made across the lower uterine segment as far from the placental edge as possible. The incision was continued down through the myometrium until a thin layer of decidua remained on the chorion. A malleable probe with a blunt tip at one end and an aperture at the other was directed through the hysterotomy incision, but outside of the membranes, toward the cervix and pulled through the vagina. The catheters, as well as implanted electrode wires, were threaded through the needle aperture, and these were exteriorized through the cervix and vagina.

After amniotomy, the fetal head or leg was delivered through the incision. If vertex presentation, a rubber bag filled with saline was placed over the head. A small amount of lidocaine was injected into the skin over the site selected for incision. Catheters were inserted into the carotid artery and jugular vein or into the femoral vessels. A silver electrode was sewn into the incision and another one on the thoracic or abdominal skin. The fetus was then replaced into the amniotic cavity and the amniotic fluid was partially replaced with warm saline. The amnion, chorion and a small portion of the decidua were then closed and an amniotic fluid catheter placed before the final suture. The uterus was then closed over all catheters and electrode wires.

A maternal femoral artery and vein also were catheterized. The animal was then placed in a restraint chair in a lateral position in a quiet room. After recovery, the animal was tranquilized with 1 mg acepromazine. The amniotic catheter was attached to a strain gauge transducer and fetal electrodes to a cardiotachometer. Fetal catheters were connected to an infusion pump and kept open with normal saline. Fetal pH, pCO_2 and pO_2 were monitored at least daily. Experiments were completed prior to signs of fetal distress.

On the morning following surgery, the chair containing the animal was placed upright while monitoring maternal arterial pressure and fetal heart rate. If there were no signs of hypovolemia, the animal was allowed to remain in the upright position and was given free access to food and water.

Cortisol, DHAS, 17β-estradiol, and ACTH were measured by radioimmunoassay [21–23].

RESULTS

Basal cortisol levels. Simultaneous fetal and maternal blood samples were drawn at different times during the day. Fetal cortisol levels were 167 ± 13.46 (mean $\pm$ S.E.) ng/ml in the morning and decreased significantly ($p < 0.001$) to 110 ± 8.9 ng/ml in the afternoon. Maternal levels showed marked variation, perhaps reflecting the presence of investigators in the room that might be masking possible diurnal fluctuation. Mean maternal levels were 325 ± 15 ng/ml.

Fetal cortisol responses to ACTH. A bolus of ACTH (0.5 IU) was administered to seven fetuses through the venous catheter. Blood samples were collected usually at 60, 30, and 15 min before, and at 5, 15, 30, 60, and 120 min after ACTH administration. Two of the fetuses tested in this fashion showed a clear increase of cortisol following stimulation with ACTH. There was no change in cortisol levels after ACTH injection in the remaining five fetuses. Figure 5 shows mean cortisol levels during ACTH stimulation in both groups of fetuses.

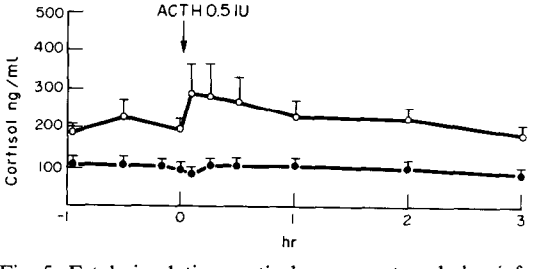

Fig. 5. Fetal circulating cortisol response to a bolus infusion of 0.5 IU ACTH in the fetal circulation. Open circles represent mean $\pm$ S.E. of cortisol levels of two fetuses that showed a response. Closed circles represent mean $\pm$ S.E. of the five fetuses that did not respond. From Serón-Ferré, M., Parer, J. T., Rose, J., Foster, D. B., and Jaffe, R. B. (Submitted for publication).

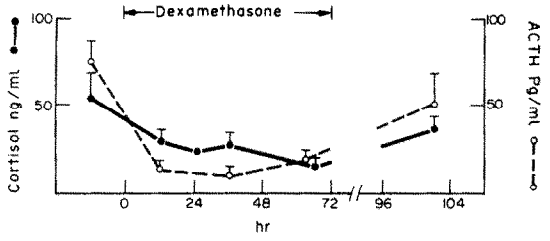

Fig. 6. Cortisol and ACTH levels in the fetal circulation of four fetuses after treatment with dexamethasone. Each point represents mean ± S.E. of four experiments; open circles: ACTH, closed circles; cortisol. From Serón-Ferré, M., Parer, J. T., Rose, J., Foster, D. B., and Jaffe, R. B. (Submitted for publication).

Fetal cortisol and ACTH levels after treatment with dexamethasone. To assess the contribution of the fetal pituitary gland to regulation of endogenous cortisol levels in the fetus, dexamethasone, 8 mg/day, was simultaneously administered directly into the fetal and maternal circulation of three pregnant monkeys. In a fourth experiment, the same dose of dexamethasone was injected into the mother. ACTH and cortisol were measured in the fetal circulation. As no difference was seen in the routes of injection of dexamethasone, the results of the four experiments were pooled. Figure 6 illustrates fetal cortisol and ACTH levels before, during and after dexamethasone treatment. As can be seen, dexamethasone readily suppressed ACTH and cortisol in the fetal circulation. After cessation of the treatment, both ACTH and cortisol returned to basal levels.

Fetal and maternal cortisol responses to ACTH challenge during dexamethasone suppression. To assess whether the fetal response to ACTH might already be maximal and therefore not responsive to exogenous ACTH, a fetus was injected with a 0.5 IU ACTH during and after cessation of dexamethasone treatment. There was no response to ACTH either during or following dexamethasone administration.

The mother of the fetus in the previous experiment was injected with 0.5 IU ACTH and fetal and maternal samples were obtained simultaneously. The purpose of this experiment was two-fold; first, to rule out a direct action of dexamethasone on the adrenal,

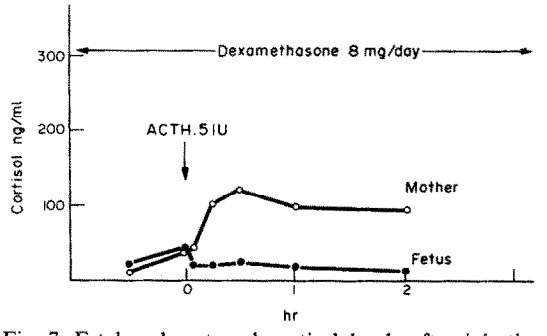

Fig. 7. Fetal and maternal cortisol levels after injection of a bolus of 0.5 IU ACTH in the maternal circulation. From Serón-Ferré, M., Parer, J. T., Rose, J., Foster, D. B., and Jaffe, R. B. (Submitted for publication).

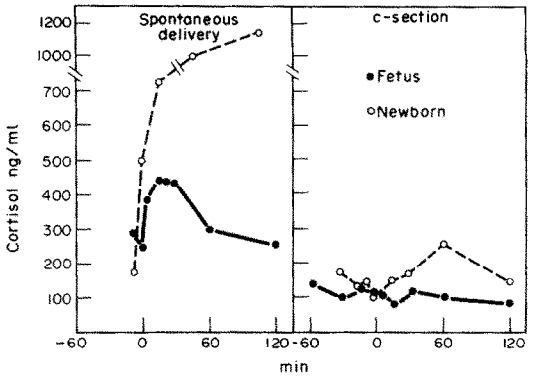

Fig. 8. Fetal and neonate response to a bolus infusion of 0.5 IU ACTH. From Serón-Ferré, M., Parer, J. T., Rose, J., Foster, D. B., and Jaffe, R. B. (Submitted for publication).

and second, to ascertain the dynamic relationship between fetal and maternal cortisol levels, when the fetus is *in utero*. Figure 7 depicts the results of this experiment. Basal cortisol levels were low in both mother and fetus, and although the mother responded to ACTH with a five-fold increase in cortisol, there was no change in fetal cortisol levels.

Comparison of cortisol responses to ACTH of the fetus and newborn. A fetus that delivered spontaneously was tested with ACTH within 12 h after delivery. Another fetus, of the same age, was tested *in utero*, delivered by cesarean section and retested 4 h after the first test. The responses to ACTH of both fetuses are shown in Fig. 8. In both cases, the newborn readily responds to ACTH with an increase in cortisol. Two other newborns were tested in a similar fashion. One of them, a premature infant delivered by cesarean section, demonstrated a response similar to the infant delivered by cesarean section illustrated in Fig. 8. The other, a term infant that delivered spontaneously, responded like the spontaneously delivered infant of Fig. 8.

Figure 9 presents a summary of the response to ACTH of seven fetuses, two newborns delivered by cesarean section, and two newborns delivered spon-

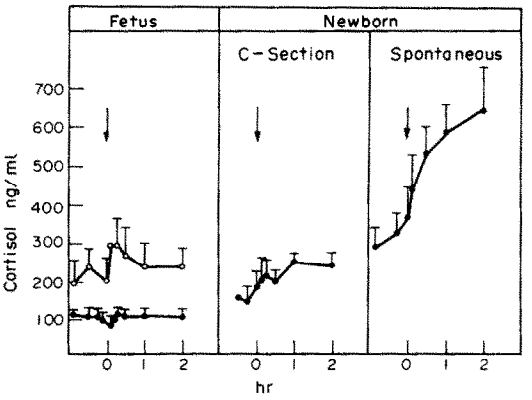

Fig. 9. Fetal and neonate circulating cortisol response to a bolus infusion of 0.5 IU ACTH. From Serón-Ferré, M., Parer, J. T., Rose, J., Foster, D. B., and Jaffe, R. B. (Submitted for publication).

taneously. There is a clear difference between the response *in utero* and *ex utero*. The amount of cortisol secreted after stimulation with ACTH increases in the newborn, and is higher in those infants that delivered spontaneously. The magnitude of the response, considered as the percent change over baseline, is similar in the three groups.

Fetal dehydroepiandrosterone sulfate. Preliminary data on DHAS response to the same experimental manipulations previously described suggest no response of DHAS to stimulation with exogenous ACTH. Dexamethasone treatment, however, readily suppresses DHAS. Maternal estradiol levels closely followed the variations of fetal DHAS.

DISCUSSION

During the last trimester, the fetal rhesus monkey *in utero* has detectable, and sometimes quite high, cortisol levels. There is a diurnal fluctuation of cortisol in the fetal circulation. This may indicate the presence of a biologic rhythm in the pituitary or adrenal or just rhythmic changes in metabolism resulting from circulatory adjustments. It is doubtful that this diurnal variation of cortisol levels in the fetus is of similar nature to the diurnal rhythm of secretion of this steroid in the adult, as circadian rhythm of cortisol production is not thought to be present in the infant [24]. Maternal cortisol levels were similar to those reported by others [25, 26]. The possible fluctuation of maternal cortisol levels may have been masked by the presence of the investigators.

The changes in fetal cortisol levels in the face of maintenance of maternal cortisol levels suggest a limited influence of maternal cortisol on fetal circulating values. This is further supported by the experiment in which an increase in maternal cortisol levels was not reflected by a change in fetal cortisol. Care must be exercised in interpreting these data, as it is possible that transfer of cortisol to the fetus may occur if maternal cortisol levels rise to higher values. Transfer of cortisol from mother to fetus has been demonstrated by Kittinger[27]. However, this experiment may not be representative of the situation in normal pregnancy, as studies were performed acutely in an exteriorized fetus. As Kittinger notes, the transfer of cortisol between the maternal and fetal circulation is determined first by the extent of placental metabolism of cortisol and, secondly, by the amount of this steroid presented to the fetal liver, and therefore susceptible to hepatic metabolism. The extent of this hepatic metabolism is important in determining the amount of maternal cortisol which reaches the fetal circulation intact. This, in turn, would be dependent upon the amount of blood coming from the placenta which is diverted through the ductus venosus directly into the right atrium of the fetus. As exteriorization of the fetus could alter the distribution of blood flow in the different fetal vascular beds [28], an exteriorized fetus may not represent adequately the situation *in utero* in regard to transfer of cortisol between mother and fetus.

The capacity of the monkey fetal adrenal gland to produce cortisol and respond to ACTH in the latter half of pregnancy has been demonstrated *in vitro* [29]. ACTH also has been shown to stimulate fetal adrenal cortisol production *in vivo* during the last third of pregnancy [30]. In our experiments, a positive response to ACTH was only observed in two fetuses, and no change in peripheral cortisol levels was seen in the remainder of the experiments. There is no discrepancy between our failure to demonstrate a response and the finding of stimulation of adrenal vein cortisol output by ACTH described by others, as the same authors note that in spite of the increased output of cortisol measured in the adrenal vein, no significant changes in cortisol concentration were seen in the peripheral circulation. The data from the dexamethasone suppression experiment indicate that the pituitary-adrenal axis is active in the monkey fetus *in utero*. ACTH and cortisol were suppressed and returned to basal levels after cessation of the infusion. This finding is in agreement with the diminished steroid levels in the mother which have been observed after dexamethasone treatment [31]. This observation is also consistent with the finding of fetal adrenal atrophy after dexamethasone treatment [32] and after hypophysectomy [33], and the fall in adrenal vein and peripheral circulating levels of cortisol after fetal decapitation [29]. These apparent discrepancies in experimental findings can be integrated conceptually if one considers that cortisol levels found in the fetal circulation are primarily of fetal origin, and that they are dependent upon the fetal pituitary. The lack of a demonstrable response to ACTH in the majority of the fetuses studies can be interpreted as the result of the fetal adrenal having a relatively limited capacity to respond to ACTH *in utero*. Thus, ACTH may be required as a tonic trophic stimulus. A maturational process may occur in the fetal adrenal gland that permits the appearance of an 'adult' response to ACTH (a burst of ACTH inducing a burst of cortisol). As different forms of ACTH or ACTH-like substances are present during fetal life [34], the possibility of dual regulation of cortisol by ACTH and other pituitary or placental peptides remains, although the lack of response to exogenous ACTH still indicates some factor limiting the response. As responses *in utero* have been observed in some of the fetuses, and as fetuses delivered by cesarean section are able to respond to ACTH, the possibility of a block existing *in utero* to the ACTH response must be considered. However, it also must be borne in mind that any change in cortisol secretion in the newborn will be modulated by the metabolic and circulatory adjustments which occur after birth.

The hypothesis of a 'maturation' of the fetal adrenal response to ACTH is supported by the finding of a larger response in the spontaneously delivered infants. As the capacity to respond to ACTH was detected

in utero in at least one of the studies, it is possible that there is a link between adrenal maturation and spontaneous labor.

Fetal cortisol levels appear to be closely controlled *in utero*. Our data demonstrate that the influence of maternal cortisol levels is limited, and that fetal cortisol is dependent on the fetal pituitary. However, the response to ACTH seen in the adult is missing during most of fetal life. Whatever the mechanism responsible (less cells sensitive to ACTH, less ACTH receptors per cell, or altered metabolism of cortisol and increased circulating volume), the fact remains that the target organs for cortisol in the fetus are protected from widely fluctuating peripheral cortisol levels. Maturation of the adrenal response to ACTH may provide the vehicle for changes in fetal maturation and the onset of labor.

III. *IN VIVO* AND *IN VITRO* STUDIES ON MONKEY FETAL TESTIS

Testosterone production by the human fetal testis during the first half of gestation has been amply demonstrated [35–37]. As there is a parallel peak of hCG and testosterone production by the fetus, it has been suggested that hCG regulates androgen formation [38]. The function of the human fetal testis during the latter part of gestation is poorly understood. In addition, little is known about the development of tropic control of fetal gonadal function. To study primate fetal testicular function and regulation during the third trimester, rhesus monkeys were studied both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Testosterone formation in vitro. Testicular tissue from fetuses was used following either cesarean section or premature delivery. Gestational ages ranged between 129 and 145 days. Testes were immediately dissected, divided into quarters, minced and preincubated in Krebs–Ringer bicarbonate glucose buffer at 37°C for 30 min, in an atmosphere of 95% O_2/5% CO_2. Thereafter, each section of tissue was incubated with NIH-hCG (10,000 IU/mg) at one of four concentrations (usually 0, 0.5, 5 and 50 μg/ml) for 3 h. At 1, 2 and 3 h of incubation, aliquots of the medium were taken for testosterone radioimmunoassay [39]. Since the antibody used had a 70% cross-reactivity with 5α-dihydrotestosterone and no attempts were made to separate this steroid from testosterone, the contamination of the testosterone values given here with 5α-dihydrotestosterone is possible.

hCG binding in vitro. Binding of [^{125}I]-hCG to testicular homogenates was determined according to the methods of Catt and Dufau[40]. Highly purified hCG (10,000 IU/mg) was enzymatically iodinated using lacto-peroxidase [41]. The specific activity of the iodinated hormone (usually 20–40 μCi/μg) was determined by measuring in the same aliquot of each preparation the maximum percentage of radioactivity bound to an excess of rat testicular hCG receptors [40] and the biological activity with a rat Leydig cell *in vitro* bioassay [42]. Testes were homogenized in 20 vol. of 50 mM Tris–HCl buffer (pH 7.3) containing 5 mM $MgSO_4$. Aliquots of the homogenate and varying concentrations of [^{125}I]-hCG were incubated in the absence and presence of a 400-fold excess of cold hCG (A.P.L., Ayerst, New York) for 16 h at 24°C. Bound and unbound hormones were separated by centrifugation.

Testosterone formation in vivo. The chronically *in utero* catheterized rhesus monkey preparations described earlier were used in the *in vivo* studies. The fetuses were challenged with intra-arterial bolus infusions of hCG (A.P.L., Ayerst, New York) and synthetic hypothalamic gonadotropin releasing hormone (GnRH). Blood samples were collected both prior to the injection of hCG or GnRH (usually at −60, −30

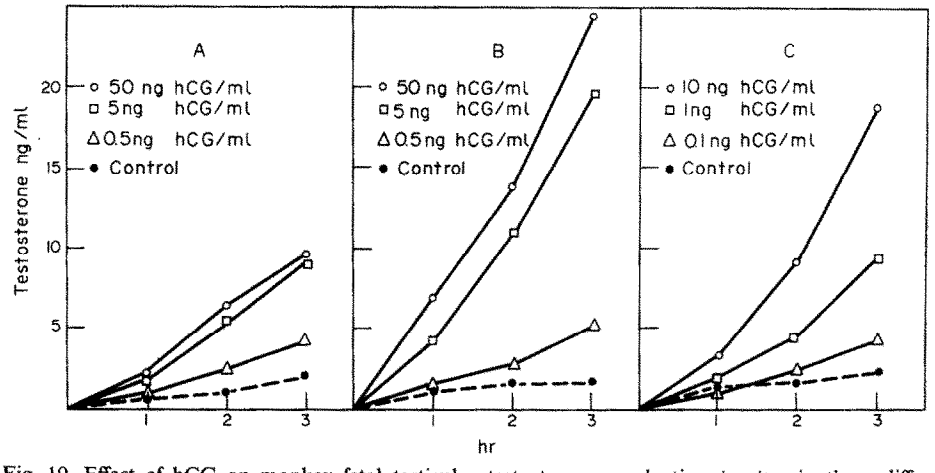

Fig. 10. Effect of hCG on monkey fetal testicular testosterone production *in vitro* in three different experiments (A, B and C). The accumulation of testosterone in the incubation medium with varying concentrations of hCG is shown as a function of time. From Huhtaniemi, I., Korenbrot, C., Serón-Ferré, M., Foster, D., Parer, J. T., and Jaffe, R. B. (Submitted for publication).

and 0 min) and after the injection (usually at +15, +30, +60 and +120 min). Testosterone was measured in the samples by radioimmunoassay.

RESULTS

Testosterone formation in vitro. A clear dose-related testosterone response to hCG in testicular incubations was seen (Fig. 10). In experiments A and B, the highest increase in testosterone output was seen between hCG concentrations of 0.5 and 5 ng/ml. The dose–responses of experiment C, using 5 times lower concentrations, are consistent with the two other experiments. Between the two highest concentrations of hCG, 5 and 50 ng/ml, the increase in testosterone output was small. Therefore, steroidogenic response of the testicular minces to hCG concentrations between 5 and 50 ng/ml was evidently maximal. The maximal production rates of testosterone in the three experiments performed were 9.6, 18.9 and 24.3 ng/mg tissue/3.

HCG binding in vitro. Binding of [^{125}I]-hCG to testicular homogenates from three monkeys was determined. In one monkey, incubations at varying concentrations of ^{125}I-hCG permitted a Scatchard analysis of the binding characteristics. The affinity constant of the binding was 1.87×10^{10}/M and the binding capacity was 102 pg/mg tissue. In the other two monkeys insufficient tissue precluded analyses at varying concentrations of hCG, and therefore only one concentration of [^{125}I]-hCG (4 ng/ml) was used. The specific hCG binding under these conditions was 31 and 43 pg/mg in the two experiments.

Testosterone formation in vivo. We demonstrated an *in utero* response in fetal testosterone production to hCG (Fig. 11). Two chronically catheterized monkey fetuses were challenged with a bolus infusion of 10 and 100 IU hCG. In both cases, a clear increase was seen in fetal serum testosterone concentrations after 15 min, and maximal response was attained in 1 h.

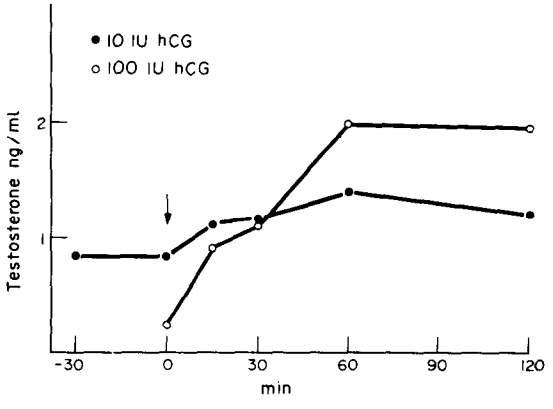

Fig. 11. Effect of bolus infusions of hCG on serum testosterone of chronically catheterized *in utero* male monkey fetuses. The arrow indicates the time of infusion. From Huhtaniemi, I., Korenbrot, C., Serón-Ferré, M., Foster, D., Parer, J. T., and Jaffe, R. B. (Submitted for publication).

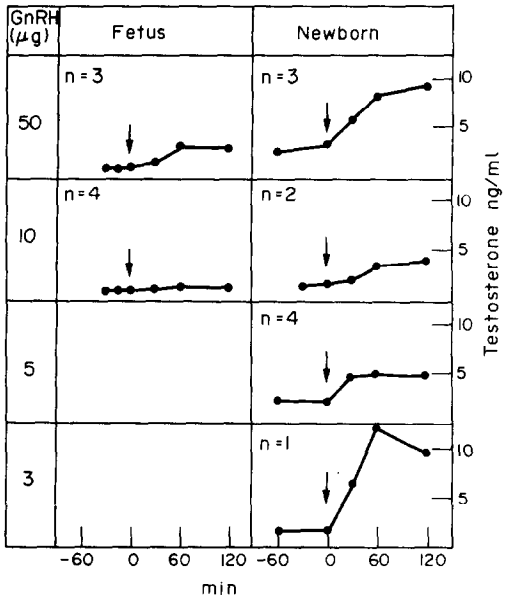

Fig. 12. Comparison of responses of serum testosterone to different doses of bolus infusions of GnRH in fetal and newborn male rhesus monkeys. The arrows indicate the time of infusions. The mean response with each dose is given and the number of animals tested with each dose is indicated by the letter *n*. From Huhtaniemi, I., Korenbrot, C., Serón-Ferré, M., Foster, D., Parer, J. T., and Jaffe, R. B. (Submitted for publication).

Other fetuses were challenged with bolus infusions of 10 μg or 50 μg GhRH (Fig. 12). With the 10 μg dose, only one out of four monkeys tested demonstrated an increase in serum testosterone concentration. The higher 50 μg dose of GnRH produced a positive response in all three animals tested, with a mean increase of 105% in plasma testosterone 1 h after the GnRH bolus.

To study possible changes in the sensitivity of the pituitary-gonadal axis after birth, we performed intravenous challenges with GnRH in neonatal male rhesus monkeys, ranging from 2 days to 6 weeks in age. In these animals, as in the fetuses, the serum testosterone concentration rose in response to GnRH (Fig. 12). In all the neonates, a positive response was seen with a dose of 3, 5 or 10 μg, while even 10 μg was ineffective in all but one fetus. The magnitude of response also increased after birth. In newborn animals, increases of up to 600% above basal testosterone concentrations were seen, while in the fetus, the maximal response observed was 200%.

DISCUSSION.

The *in vitro* studies performed demonstrate the steroidogenic activity of the fetal monkey testis during the last third of gestation. Furthermore, the testes were able to specifically bind iodinated hCG, and this gonadotropin was able to stimulate testosterone biosynthesis. The binding capacity of the testicular homogenate (102 pg/mg) was about three times higher than that observed in human fetal testes of the second

trimester [43]. The association constant (1.87×10^{10}/M) was similar to those observed for testicular LH/hCG receptors in other species [40, 43–45]. hCG stimulated the highest increase in testosterone formation in the testicular minces between concentrations of 0.5 and 5 ng/ml. Increasing hCG concentration from 5–50 ng/ml did not clearly increase testosterone production, which was evidently maximal at the lower, 5 ng/ml, concentration. Maximal testosterone production was achieved at the same concentrations in the human fetal testes of 14–18 weeks gestational age [43].

How these observations on monkey fetal testicular activity correlate with the human fetus of the third trimester is not known. Indirect evidence, based on the observed hypoplasia of genitalia of male anencephalic newborns, suggests that human fetal testes also are active during the latter part of gestation and stimulate genital growth [46]. This also suggests that fetal pituitary function is needed for the normal function of gonads during the latter part of gestation. Similar results have been obtained in rhesus monkeys in which hypophysectomy of the fetus significantly reduces testicular weight [47]. This picture is, however, complicated by the fact that levels of chorionic gonadotropin are high enough throughout gestation in human fetal circulation to maintain testicular steroidogenesis [46, 48]. In monkey pregnancy, no measurable chorionic gonadotropin is present after the first third of gestation [49, 50]. Thus, if monkey fetal gonads are under gonadotropic stimulation near term, the source of this stimulation is most likely the fetal pituitary.

From the *in utero* studies, further evidence for testicular responsiveness to gonadotropic (hCG) stimulation was obtained. Furthermore, evidence for the role of the fetal pituitary in this stimulation was obtained as GnRH was effective in stimulating an increase of fetal plasma testosterone levels. This effect presumably was mediated through stimulation of LH release from the fetal pituitary, which then stimulated testicular steroidogenesis. The LH levels after GnRH infusions have not been measured as yet. The observations do, however, suggest the presence of a functional pituitary-gonadal axis in the male fetal monkey during the last third of gestation.

Only the high dose (50 μg) of GnRH was able to stimulate an increase in fetal plasma testosterone levels consistently. In contrast, in the newborn, doses as low as 3 μg were effective. Further, while the maximal increase in plasma testosterone was about 200% *in utero*, stimulations of up to 600% were seen after birth. Thus, both the sensitivity and the magnitude of the response increase after birth. This difference may be explained, in part, by changes of blood vol. and kinetics of GnRH, LH and/or testosterone after birth. Another explanation is a change of sensitivity in target organs, either the pituitary or testes. A change in pituitary sensitivity after birth could well be expected with the elimination of the negative feedback action of some placental hormones (perhaps progesterone or estrogens). Exploration of these perinatal endocrine changes might yield valuable information about the development of the hypothalamic-pituitary-gonadal axis.

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DISCUSSION

Naftolin. There are two things that come to mind. In the light of your studies do you see any reason to suspect the presence of placental or other ACTH? Secondly you showed us changes in cortisol and testosterone blood concentrations. Is it possible that either or both of these are not coming from the "unusual" organs, such as the fetal lung?

Jaffe. In answer to your first question we are indeed looking for the factor responsible for decreased sensitivity prior to delivery. There are factors in pregnancy which may be responsible for the "blocking" effect that we see. We have several candidates for this. One of the prime ones, in view of the recent work of my colleague, Dr. Siiteri, is progesterone itself, and we are in the process of starting to look at placental extracts as well as individual steroidal and protein hormones to see whether these exert an effect. In answer to your second question, of course we can't exclude other sites for steroid production and metabolism. The fact that the *in vitro* studies we have performed with both the adrenal and testis in the human and the monkey correlates so well with our *in vivo* studies suggests to us that at least these glands are the primary sites of production. In regard to testicular regulation, one of the things that we didn't think to look at yet, which we now will, is what happens in the female fetus in terms of testosterone production, when we stimulate it with hCG. We have those samples. It is just a question of performing the assays.

Challis. We also have some evidence in the pregnant monkey for an increase in the secretion of androstenedione and in fact of progesterone by the fetus in late pregnancy. I wonder if you can give us any information about the specificity of the HCG binding in your human fetal adrenal studies and whether there is any interaction with HCH and ACTH binding sites.

Jaffe. We have not performed HCG binding studies in the fetal adrenal as yet. We have only done the superfusion studies.

Oakey. I would agree with Dr. Jaffe that the time one tends to study the human fetal adrenal, between 12 and 20 weeks, is the time when things are likely to be changing. When we were looking at the biosynthesis of steroids from acetate by incubations of this kind of tissue we found that ACTH consistently stimulated the incorporation of acetate into D sulphate whereas the only tissue sample we tried with HCG failed to show any stimulation of incorporation. That was a piece of tissue from an adrenal that showed stimulation with ACTH.

Jaffe. I am aware of those studies, Dr. Oakey, but my understanding was that whereas you did not demonstrate acetate incorporation into dehydroepiandrosterone sulfate, you did demonstrate acetate incorporation into cholesterol in those studies, am I remembering correctly?

Oakey. We found incorporation into cholesterol, D sulfate and pregnenolone sulfate, that is steroids of the 5-ene-pathway.

Jaffe. But with HCG did you not find acetate incorporation into cholesterol?

Oakey. Incorporation into cholesterol, but this was not stimulated by HCG. In searching for other stimulatory factors have you tried human growth hormone? To our surprise this gave a really tremendous stimulation to the incorporation of acetate into pregnenolone, pregnenolone sulfate, dehydroepiandrosterone and its sulfate.

Jaffe. Dr. C. H. Li in our school was kind enough to furnish us with growth hormone which we have just superfused but haven't analyzed the results. Similarly, Dr. Friesen has recently provided us with a preparation of prolactin which we will also be using.

Solomon. Dr. Oakey and I are coming up there with some different results but I have looked at your papers rather carefully and they don't agree with anything we've published previously. One of the things I haven't been able to find in your papers is the percentage conversion of acetate to products? I wonder if you can tell us what types of conversion you are working with in view of the fact that you need 100 milligram amounts of steroid forming with pregnancy.

Oakey. We did not publish the percentage conversion to product because with a substrate like radioactive acetate one incubates several tens of microcuries. We feel it is really irrelevant when one finishes up with several thousand d.p.m. of a particular steroid product well down the biosynthetic chain. What we were concerned to show was whether particular steroids such as 5-ene-3β-hydroxysteroid sulfates were being formed and whether under the same conditions we could also detect synthesis of the 4-ene-3-oxo steroids such as cortisol. Using the same kind of criteria for both groups we could readily detect only the 5-ene-3β-hydroxysteroids and their sulphates and recrystalize them to constant isotope ratio. The recrystalization figures were not published in the Journal because the editors said we couldn't take up 7 pages with tables of recrystalizations. We have convinced ourselves anyway, that 5-ene-3β-hydroxy steroids are being formed from acetate.

Jaffe. Without quantitative data, the ability of labeled material to be converted to labeled material only gives us an idea of the capacity of the tissue and does not furnish the information that's required in regard to how much endogenous material is really produced.