Ph.D. ABSTRACT

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ACTION OF HUMAN CHORIONIC GONADOTROPHIN (HCG) ON STEROID SYNTHESIS IN PLACENTAL AND ADRENAL TISSUES

The action of HCG on pathways of steroid biosynthesis was studied 'in vitro' using different tissues. In placental minces, testosterone aromatization to estrone and estradiol was increased by NADP, further enhanced by the simultaneous addition of HCG and/or cyclic 3′,5′-AMP, but not to the same extent as following addition of G-6-P. The effect of HCG required intact cells and was only observed after a lag period of one to two hours. In the absence of NADP, HCG and cyclic 3′,5′-AMP inhibited aromatization. In adrenal tissues from either newborn infants or adult rats, HCG inhibited 3β-hydroxysteroid dehydrogenase as well as 11β-,16α-, 18-,19- and 21-hydroxylases. The data suggest that the action of HCG in the feto-placental unit differs, depending on the target organ; it stimulates estrogen synthesis in the placenta, but inhibits corticosteroid formation in the adrenals.

SHORT TITLE

ACTION OF HCG ON STEROID SYNTHESIS IN PLACENTAL AND ADRENAL TISSUE

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ACTION OF HUMAN CHORIONIC GONADOTROPHIN (HCG) ON STEROID SYNTHESIS IN PLACENTAL AND ADRENAL TISSUES

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LIST OF ABBREVIATIONS USED

ACTH	adrenocorticotrophic hormone
cyclic 3′,5′-AMP or 3′,5′-AMP	adenosine-3′,5′-cylic monophosphate
3′,5′-AMP dibutyryl (D.B)	2′-dibutyryl 3′,5′-cyclic adenosine-monophosphate-sodium
c, mc, μc	curie, millicurie, microcurie
c.p.m.	counts per minute
d.p.m.	disintegrations per minute
G-6-P	glucose-6-phosphate
gm, mg, μg	gram, milligram, microgram
hr	hour
g	gravitational force
HCG	human chorionic gonadotrophin
I.U.	international units
KRBG	Kreb's-Ringer bicarbonate glucose solution
ml	millilitre
min	minute
M	molar
mmol, nmol, μmol	millimole, nanomole, micromole
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
TLC	thin layer chromatography
S.A.	specific activity
R_f	mobility relative to the solvent front
Δ^4 or Δ^5	double bond between C-4 and C-5 or C-5 and C-6

pregnenolone	3β-hydroxypregn-5-en-20-one
17α-hydroxy-pregnenolone	3β, 17α-dihydroxypregn-5-en-20-one
16α-hydroxy-pregnenolone	3β, 16α-dihydroxypregn-5-en-20-one
progesterone	pregn-4-ene-3,20-dione
17α-hydroxy-progetserone	17α-hydroxy-pregn-4-ene-3,20-dione
deoxycorticosterone (DOC)	21-hydroxy-pregn-4-ene-3,20-dione
corticosterone (B)	11β,21-dihydroxy-pregn-4-ene-3,20-dione
aldosterone (Aldo)	11β,21-dihydroxy-pregn-4-ene-3,20-dione-18-al
cortisol (F)	11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione
18-hydroxy-11-deoxy--corticosterone (18-OH-DOC)	20, 21-dihydroxy-18,20-epoxy-pregn-4-en-3-one
dehydroisoandrosterone (DHA)	3β-hydroxyandrost-5-en-17-one
16α-hydroxy-dehydroisoan--drosterone (16α-OH-DHA)	3β,16α-dihydroxyandrost-5-en-17-one
testosterone	17β-hydroxy-androst-4-en-3-one
androstenedione (ASD)	androst-4-ene-3,17-dione
11β-hydroxy-androstene--dione (11β-OH-ASD)	11β-hydroxyandrost-4-ene-3,17-dione
16α-hydroxy-androstene--dione (16α-OH-ASD)	16α-hydroxyandrost-4-ene-3,17-dione
19-hydroxy-androstenedione (19-OH-ASD)	19-hydroxy-androst-4-ene-3,17-dione
19-hydroxy-testosterone (19-OH-TESTO)	17β,19-dihydroxy-androst-4-ene-3-one
estrone (E_1)	3-hydroxyestra-1,3,5(10)-triene-17-one
estradiol (E_2)	estra-1,3,5(10)-triene-3,17β-diol
estriol (E_3)	estra-1,3,5(10)-triene-3,16α,17β-triol
pregnenolone sulfate	20-ketopregn-5-ene-3β-ylsulfate
17α-hydroxy-pregnenolone sulfate	17α-hydroxy-20-ketopregn-5-ene-3β-yl sulfate
dehydroisoandrosterone sulfate (DHAS)	17-ketoandrost-5-ene-3β-yl sulfate

16 α -hydroxy-dehydroisoand--rosterone sulfate	16 α -hydroxy-17-ketoandrost-5-ene-3 β -yl sulfate
testosterone sulfate	3-oxoandrost-4-en-17 β -yl sulfate
testosterone acetate	17-acetoxy-androst-4-en-3-one
19-hydroxy-testosterone diacetate	17 β ,19-diacetoxy-androst-4-ene-3-one
19-hydroxy-androstenedione acetate	19-acetoxy-androst-4-ene-3,17-dione
16 α -hydroxy-androstene-dione acetate	16 α -acetoxy-androst-4-ene-3-1-one
estrone monoacetate	3-acetoxy-estra-1,3,5(10)-triene-17-one
estradiol-17 β -diacetate	3,17 β -diacetoxy-estra-1,3,5(10)-triene
adrenosterone	androst-4-ene-3,11,17-trione
20 α -dihydroprogesterone	20 α -hydroxypregn-4-en-3-one

I . REVIEW OF THE LITERATURE

The concept of the human feto-placental unit has been much docu--mented in recent years. According to this concept, the fetus and placenta form a functional unit which carries out the enzymatic reactions involved in the biosynthesis of a number of steroid hormones. Estrogens represent quantitatively the principal end products of such reactions during pregnancy (1). Since the enzymes lacking in the placenta are present in the fetus, the fetus and placenta together are able to synthesize estrogens from simple precursors. Because of this special distribution of enzymes, the steroid precursors shuttle back and forth from fetus to placenta in the successive steps of estrogen biosynthesis (2).

As a result of the great attention focused in recent years on steroid metabolism in the human fetus and placenta, a vast amount of liter--ature has been accumulated. For a full discussion on this subject the reader is referred to many review articles, particularly those by Diczfalusy et al and Solomon et al (3-16). The literature pertaining more specifically to experiments that are reported in this thesis will be discussed.

Section I of the review will be devoted to biosynthesis and meta--bolism of steroid hormones in the placenta. This section also will encompass studies that have been reported with human chorionic gonadotrophin (HCG), a protein hormone secreted by placenta. This inclusion has been made in an attempt to give our studies a proper background against which our results can be discussed from a comparative point of view.

Section II will deal with the biosynthesis and metabolism of steroids in the human fetus. Attention will be focused mainly on the fetal adrenal. The action of adrenocorticotrophic hormone (ACTH) and HCG will also

be discussed here.

Section III will be devoted to the main pathways involved in the biosynthesis of estrogens in the feto-placental unit. Consideration will be given to factors which control the synthesis of estrogens in pregnancy.

SECTION I.

Studies with placenta:

A new organ, the placenta is developed soon after fertilization, and its primary function is to sustain the nutrition and growth of the developing embryo. In addition to this, the human placenta is also the site of production of such hormones as estrogens, progesterone, chorionic gonado--trophin, etc., which are essential for the maintenance of pregnancy. The endocrine function of the placenta had been postulated by Halban as early as 1904 (17,18). In 1922 Allen developed a bioassay (19) which provided the basis of measurements of estrogenic activity in placental tissue. Progestational activity was detected in extracts of human placental tissue, as early as 1932 (4). Twenty years later it was shown (21-23) that placental extracts contain progesterone, the same gestagen previously found in the corpus luteum. In 1930, Philip (24) postulated that the placenta was the main source of urinary gonadotrophin during pregnancy. The studies by Jones et al (25) established the placental origin of this tropic hormone even more firmly.

Soon after conception, the human zygote divides repeatedly as it traverses the fallopian tube. By the time the zygote has reached the uterine cavity (three to four days after fertilization) it is a ball of cells (morula). Within the uterine cavity the morula draws nourishment and oxygen from the uterine secretions. The uterine fluid is imbibed and a portion accumulates between the cells, eventually causing the separation of an outer layer of flattened cells (the trophoblast), and an inner mass of cells (the embryo), attached to the trophoblast in one small area. Thus we have the very

begining of visible differentiation of placenta versus fetus. During this process of embedding within the uterine mucosa, the trophoblast becomes active and differentiates into two types of cells; an inner cytotrophoblast, with distinct cell boundaries and an outer syncytiotrophoblast, with no cell boundaries (26). Even at this early stage of development (nine days), it is possible to detect human chorionic gonadotrophin (HCG) in maternal urine (27), a protein synthesized by the trophoblast (25,28-30). By the early part of the fifth month, the placenta completes its period of morphogenesis. There is continued growth of the placenta during the second half of pregnancy, the placenta at term weighing about 650 gm ; but the general structure of placenta remains unchanged after the fifth month (31).

Placental metabolism of acetate and cholesterol

In 1965, Van Leusden and Villee (32) showed that minces of term placenta can synthesize squalene and small amount of cholesterol from acetate. In 1966, Zelewski and Villee (33) showed that minces of term placenta could utilize mevalonate and acetate in the synthesis of squalene, lanosterol and cholesterol. A minute conversion of acetate to cholesterol was also demonstra--ted by Levitz et al (34) in 'in vitro' perfusion of human placenta. In 1970, experiments of Telegdy et al (35-37) showed that when midgestation placentas as well as complete feto-placental units were perfused with millicurie amounts of ^{14}C-acetate and ^{3}H-cholesterol, the midterm placentas were incapable of steroid synthesis from acetate.

These findings of Telegdy et al were confirmed by Mathur et al (38) indicating that at midgestation, the placenta does not synthesize cholesterol to any significant extent.

In 1954, it was first reported by Solomon et al (39) that the human placenta perfused 'in vitro' was capable of converting cholesterol to pregnenolone. In 1965, Jaffe et al (40) reported that 'in situ' perfusion of midterm placenta with cholesterol resulted in the formation of pregne--nolone. In the following year, Jaffe and Peterson (41) confirmed this conversion using term placenta.

The biosynthesis of progesterone from cholesterol was demonstrated by Solomon et al (42) in 'in vitro' perfusion of placenta. Maeyama et al (43) have also shown the 'in vitro' conversion of cholesterol tn progesterone by the placenta at term. 'In vitro' conversion of cholesterol to pregnenolone and progesterone was shown by Morrison et al (44) using a placental mitochondrial acetone powder.

Placental metabolism of pregnenolone:

The conversion of pregnenolone to progesterone was first reported by Pearlman et al (45) in 1954, in homogenates of placenta at term. Sobrevilla et al (46) showed that the incubation of term placental homogenates with pregnenolone resulted in the formation of progesterone and in small amounts of 20α-dihydroprogesterone. Midterm placentas perfused 'in situ' with pregnenolone and 17α-hydroxypregnenolone (47) converted these steroids to progesterone and 17α-hydroxyprogesterone respectively. Jaffee and Ledger (48) isolated progesterone from term placentas following 'in situ' perfusion with pregnenolone. It is well established that at midterm (49) as well as at term (50) the placenta perfused 'in situ' with pregnenolone sulfate,hydrolyzes major quantities of this compound. When Solomon et al (10) injected pregne--nolone and pregnenolone sulfate into the umblical vein, they were able to demonstrate progesterone and 20α-dihydroprogesterone in the placenta, but not in the fetal tissue; suggesting that the 3β-hydroxysteroid dehydrogenase

and Δ^5-isomerase were active only in the placenta. Furthermore, when pregnenolone sulfate was infused into term placenta 'in situ', the conversion of this compound to progesterone was confirmed.

Following incubation of placental homogenates with 17α-hydroxy--pregnenolone, Jungmann and Schweppe (51) were able to isolate both dehydro--isoandrosterone and androst-5-ene-3β,16α,17β-triol as well as some androstenedione and testosterone. The authors concluded that the enzyme$_{17\text{-}20}$, desmolase was present in the placenta. However after perfusion of the placenta 'in situ' with C-21 precursors (47,52,53), the activity of this enzyme could not be demonstrated. Pion et al (47) was equally unable to detect any C-19 steroids following the 'in situ' perfusion of placentas with pregnenolone and 17α-hydroxypregnenolone.

By the incubation of midterm placenta, it was possible to demonstrate the presence of 16α-hydroxylase and 17α-hydroxylase in the placenta (54,51). Once again the results obtained from 'in situ' perfusion of placenta could not show any 16α, or 17α-hydroxylated steroids (10,55,45-48,56). However, the presence of 6β-hydroxylase in placental tissue was confirmed. Following the perfusion of term placentas 'in situ' with pregnenolone, Jaffe and Ledger (48) were able to isolate progesterone and 6β-hydroxyprogesterone.

Placental metabolism of progesterone:

The 'in vitro' conversion of progesterone to androstenedione has been demonstrated in the homogenates of term (57) and midterm(51) placenta, and localized in the 105,000 X g supernatant of the subcellular fraction of placenta (58). The conversion of progesterone to testosterone has also been reported in the homogenates of midterm placenta (51). On the otherhand

Sobrevilla et al (46) despite a careful search, could not identify androstenedione or testosterone, following the incubation of term placental homogenates with 17 α -hydroxy-progesterone. Similar negative findings were recorded in the placenta or perfusate when midterm or term placentas were perfused with progesterone 'in situ' (47,52,53). Even the experiments of Jaffe et al (59), in which progesterone and androstenedione each bearing a different label were introduced into the intact feto-placental circulation, indicate that little, if any, androstenedione is formed from progesterone by the placenta or by the fetus.

The 'in vitro' conversion by placental tissue of progesterone to 20 α -dihydroprogesterone (60) and of 17 α -hydroxy-progesterone to 17 α , 20 α -dihydroxypregn-4-en-3-one (46) has been demonstrated. The latter reaction was also observed in 'in vitro' perfusion experiments (61). 'In situ' perfusion experiments at mid pregnancy indicate that large amounts of 20 α -dihydroprogesterone are converted to progesterone, but only very small quantities of progesterone are converted to 20 α -dihydroprogesterone (62,52). On the other hand, there is no interconversion in the placenta between 20 β -dihydroprogesterone and progesterone (52). These findings are in close agreement with the histochemical data of Hart (63), who could not demonstrate any 20 β -hydroxysteroid dehydrogenase activity in midterm or term placentas.

Placental metabolism of corticosteroids

The data available suggest that with the exception of 11 β --hydroxysteroid dehydrogenase, enzymes which are involved in the oxidative metabolism of corticosteroids do not seem to be present in the human placenta (12). It has been convincingly demonstrated that 11 β -hydroxy-corticosteroids

are converted in a good yield to 11-oxo-steroids when incubated with homogenates of term placenta (64). Furthermore, the concentration of cortisone is much higher than the concentration of cortisol in extracts of term placenta (65).

Placental metabolism of dehydroisoandrosterone, androstenedione, and testosterone:

Considerable amounts of dehydroisoandrosterone sulfate and some dehydroisoandrosterone reach the midterm placenta from the fetal as well as the maternal circulation (66,67). Baulieu et al (68) reported the formation of testosterone from dehydroisoandrosterone with homogenates of placenta. Bloch and Newman (69) showed the conversion of dehydroisoandrosterone to androstenedione, using slices of placentas. The midterm placentas perfused 'in situ' could also convert dehydroisoandrosterone (70,71) as well as dehydroisoandrosterone sulfate (71,72) to androstenedione and testosterone. Dell'Acqua et al (70) showed that placentas perfused 'in situ' can incorporate 16α -hydroxy-dehydroisoandrosterone into 16α -hydroxy-androst-4-ene-3,17-dione. A slight 7-hydroxylating ability of human chorionic tissue has also been shown 'in vitro', using dehydroisoandrosterone as a substrate (73). The available data indicate that the placenta converts considerably more testo--sterone to androstenedione than vice versa (74-76).

It appears that little, if any, conjugation of dehydroisoandrosterone (74) takes place in the placenta. Placental conjugation of testosterone has not yet been demonstrated. Also little, if any, testosterone sulfate is hydrolized by human placenta 'in vitro'(77), or 'in vivo'(78). On the other--hand, hydrolysis of dehydroisoandrosterone sulfate in human placental preparations has been shown both 'in vitro' (79,80) and in perfusion studies carried out 'in situ' (81,74) or following the administration of this conjugate into a uterine artery at laparotomy (82).

Since the pioneer work of Meyer (83) and Ryan (84,85), a vast amount of information has accumulated on placental aromatization of the steroid molecule. Ryan (86) demonstrated for the first time,the ability of human placental microsomes (80,000 X g) to aromatize various neutral C-19 steroids to estrone and estradiol, when reduced nicotinamide adenine dincleotide phosphate (NADPH) and oxygen were present. Testosterone was converted by this placental preparation to estradiol and androstenedione to estrone in high yield. The conversion of dehydroisoandrosterone to estrone was also demonstrated. However, this conversion of 3β-hydroxy-Δ^5-steroids to estrogens occurs, only when they can be first converted to Δ^4-configuration (87,88). Morato et al (89) extended these studies by demonstrating the conversion of not only dehydroisoandrosterone, but also dehydroisoandrosterone--sulfate to estrone by a placental microsomal preparation. Wu et al (90) confirmed the conversion of dehydroisoandrosterone to estrone and estradiol in homogenates of placenta. Perfusion of term placentas 'in vitro' through both fetal and maternal circulations with dehydroisoandrosterone sulfate and testosterone, resulted in the formation of estrone, estradiol, androstenedione and testosterone from both substrates (91).

In spite of the fact placenta contains large amounts of estriol, no direct evidence for the 'in vitro' synthesis of this steroid by this organ has as yet been provided. Incubation of testosterone with term (92), as well as midterm (93) placentas, didnot yield estriol. 'In situ' perfusion of midterm placentas with labelled dehydroisoandrosterone and/or its sulfate, androstenedione and testosterone, invariably indicated the absence of estriol from the placenta and perfusate, although large quantities of estrone and estradiol were isolated from both sources (81,94,70,71). On the other hand 16-hydroxylated steroids such as 16α-hydroxy-testosterone, 16-hydroxy-

-androstenedione (95) and Δ^5-androst-3β-16α, 17β-triol (96,95), were shown by Ryan to be readily converted to estriol by placental preparations. This suggests that, 16-hydroxylated steroids may be of physiological importance in the formation of estriol by the placenta.

In view of these data, it can be concluded that the human placenta is unable to carry out 16α-hydroxylation of estrogens. This reaction which is required for the formation of estriol must take place in extraplacental sources, such as the fetal and maternal organisms (12).

Placental metabolism of estrogens

There is a rapid interconversion between estrone and estradiol in placentas perfused 'in situ' ; generally more estradiol being present in the placenta and more estrone in the perfusate (81,71). The incubation of estrone and estradiol with slices of term and midterm placentas (97), failed to pro--duce estriol; confirming the lack of 16-hydroxylation in the placenta. Similarly, no estriol could be demonstrated following the perfusion of term placental cotyledons with estrone and estradiol (98).

As we have seen above, the placenta is a source of HCG during pregnancy. HCG is a glycoprotein and has a high content of carbohydrate (25%) which appears to be necessary for the hormone action (99).

It has been shown that HCG appears in maternal urine very early after implantation of the fertilized ovum and reaches peak value of 20,000-40,000 I.U. per day between the 50th. and 70th. days after the first day of the last menses. In the third to fourth lunar month, there is an abrupt decrease in the amount excreted. From the fifth month onward,the concentration remains at a fairly constant level ranging from 3,000 tol0,000 I.U. per day. After delivery, HCG disappears from the maternal urine within a few days (100). Serum concentrations of HCG during the course of pregnancy, yields curves similar to those of urinary

excretion (101). Following expulsion of the placenta, the hormone disappears from maternal serum within 48 hours.

The studies carried out by Diczfalusy and Tögren (102), showed concentrations of 450 I.U. per gm. of placental tissue during the second or third months of gestation. In the fourth lunar month an abrupt drop in the concentration was observed, and from the fifth month onward this remained at a fairly constant level until term. Thus the levels of HCG found in the placenta throughout pregnancy, parallel the concentrations observed in the urine and serum. These results suggest that the hormone is not being stored, but is being released continuously into the circulation (100).

Very little is known about the secretion of HCG. Neither the maternal pituitary, nor the fetus appears to affect the secretion of HCG (100,103). Mcreover the function of HCG in human pregnancy remains obscure, although its high titer has been used for the diagnosis of pregnancy. Analysis of ovarian venous blood (104) as well as 'in vitro' studies (105,106) indicate that the corpus luteum continues to produce progesterone and other steroids during pregnancy. HCG can prolong the life span of the corpus luteum (107), causing it to continue to secrete progesterone and estrogens (100), but again the role of HCG when it reaches its maximum production is not clearly understood. On the basis of clinical observations it has been postulated that HCG may stimulate the production of estrogens and gestagens by the placenta as it does in the ovary (103). However during pregnancy it has been shown that these steroids reach their maximum concentrations in blood and urine, not in the second or third lunar months like HCG, but in the last trimester (103). The question arises why the placenta produces such large amounts of HCG in the second and third lunar months, and considerable

quantities thereafter. In this context, it has been suggested that estriol may stimulate the output of chorionic gonadotrophin (108).

Cedard et al (109) in an attempt to explain the role of HCG in estrogen biosynthesis by the placenta, carried out 'in vitro' perfusion studies. They were able to demonstrate that 20,000 I.U. of HCG added to the perfusion medium significantly improved the yield of estrone and estradiol from C-19 neutral steroids, i.e., from androstenedione, dehydroisoandrosterone, 3β, 17β-dihydroxy-androst-5ene, and testosterone, whereas the production of estrone and estradiol from 19-hydroxy-androstenedione was not modified.

They investigated further the action of HCG in the placenta obtained during the first trimester of pregnancy, when HCG elimination is maximal. In control experiments, the ability of the placenta to aromatize the testosterone molecule was small, but the addition of HCG (or LH), induced a significant increase in estrogen production (110,111). Measuring the glucose and lactic acid concentrations in the perfusion fluid of term placentas, they showed that the addition of HCG did not modify lactic acid concentrations but induced a statistically significant, though transitory increase in glucose levels in the liquid (111).

They also observed that the addition of a NADPH-generating system or of only 500 mg Glucose-6-phosphate (G-6-P) increased the estrogenic production from testosterone as markedly as HCG addition. Similar observa--tions were recorded when only adenosine-3', 5', cyclic monophosphate (3',5'-AMP) was added (111).

Earlier Toren and Gordon (112) demonstrated in perfusion studies with placenta, that when HCG was added to the perfusing fluid, there was a significant disappearance of citrate. This reaction was further stimulated by addition of both estradiol and HCG to the perfusing fluid.

These results suggested that HCG may act like ACTH in the adrenal cortex or like luteinizing hormone (L.H.), in the corpus luteum to eventually produce increased amounts of NADPH and accelerate in this way the hydroxylation and aromatization (111).

SECTION II.

Studies with human fetus and newborn (with particular reference to the adrenal)

The fetal adrenal is composed of two distinct zones of tissue; an inner 'fetal' zone and an outer 'definitive' zone. The fetal adrenal reach their maximum size by the fourth month of gestation, when they are as large as the kidney. Their size declines slowly until birth after which time the 'fetal' zone disappears by degenerative involution (113).

Fetal metabolism of acetate and cholesterol

In incubation studies of midterm fetal adrenal slices, Bloch and Benirschke demonstrated the conversion of acetate to dehydroisoandrosterone, androstenedione, pregnenolone, 11β-hydroxy-androstenedione, and cortisol (114), as well as that of acetate to cholesterol (115,116). Villee et al (117) could not demonstrate the conversion of acetate, mevalonate and choles--terol to neutral steroids, by slices and homogenates of fetal adrenal. Incubating labelled acetate in an organ culture of fetal testes for eight days, Rice et al (118) were able to isolate pregnenolone, progesterone, 17α-hydroxy-progesterone and small amounts of androstenedione and testosterone from the medium.

Perfusion studies carried out at 20^{o}C indicated that the midterm fetal adrenal and liver were able to convert some acetate to cholesterol (10), but neither the acetate nor the cholesterol was converted in appreciable

quantities to neutral steroids. When perfusion studies were performed at 35-36°C by Telegdy et al (35-37), as well as by Mathur et al (38), there was a conversion of acetate to cholesterol by the midterm fetal liver, adrenal, residual fetal tissue and perfusate. Telegdy et al however observed, the formation of pregnenolone and dehydroisoandrosterone in the perfusates, liver, and adrenal of the human fetus as major products of acetate and cholesterol. These authors concluded that the fetus can synthesize cholesterol from acetate and is also capable of de novo synthesis of pregnenolone and dehydroisoandrosterone.

Fetal metabolism of pregnenolone

Both pregnenolone and pregnenolone sulfate are present in cord blood (119,120). 'In vitro' conversion of pregnenolone to 16α-hydroxy--pregnenolone and dehydroisoandrosterone has been reported by Villee et al (121), using homogenates and slices of fetal adrenal. Villee and Driscoll (122) reported the conversion of pregnenolone to androstenedione and 11β--hydroxy-androstenedione by minces of adrenals from fetuses. Furthermore, when pregnenolone was incubated with adrenal slices from an anencephalic newborn, it was converted to progesterone and 16α-hydroxy-dehydroisoandroste--rone(123). Milner and Mills (124) incubated pregnenolone with adrenal slices of human fetuses and demonstrated its conversion to dehydroisoandrosterone, dehydroisoandrosterone sulfate, androstenedione, 11β-hydroxy-androstenedione, cortisol and 11-deoxycortisol.

In contrast to the 'in vitro' studies, efforts to demonstrate the conversion of pregnenolone to α,β-unsaturated, 3-ketosteroids, following injection of pregnenolone into the umbilical vein at midterm were unsuccessful (10). In these studies however, Solomon et al (10) were able to isolate from adrenals pregnenolone sulfate, 17α-hydroxy-pregnenolone sulfate,

dehydroisoandrosterone sulfate, and dehydroisoandrosterone.

Jackanicz et al (125) have demonstrated the conversion of 17α -hydroxy-pregnenolone to cortisol and 17α -hydroxy-progesterone, by the adrenals of previable fetuses perfused 'in vitro'. Following the injection of labelled 17α -hydroxy-pregnenolone or 16α -hydroxy-pregnenolone into the umbilical vein, Reynolds et al (126) were able to isolate either cortisol and dehydroisoandrosterone, or 16α -hydroxy-progesterone respectively from the adrenals of midterm fetuses. Maeyama et al (127) injected pregnenolone into the umbilical vein of anencephalic infants after delivery and have tentatively identified dehydroisoandrosterone in the adrenals.

'In vitro' perfusion of previable fetuses with 17α -hydroxy-pregnenolone has been shown to result in the formation of 17α -hydroxy-pregnenolone sulfate and dehydroisoandrosterone sulfate in the adrenals (128). Perez-Palacios et al (129) reported that fetal adrenal homogenates can convert pregnenolone sulfate to 17α -hydroxy-pregnenolone sulfate, dehydroisoandrosterone sulfate and 16α -hydroxy-dehydroisoandrosterone sulfate.

Fetal metabolism of progesterone

The studies of cord blood show a most impressive arterio-venous difference in the concentration of progesterone, indicating that major quantities of progesterone are secreted by placenta to the fetus (130). It is well established that progesterone is extensively metabolized by the fetus (131).

Fetal adrenal homogenates can convert progesterone to androstene-dione (132), 17α -hydroxy progesterone (132,117), 16α -hydroxy-progesterone (117,122), deoxycorticosterone (117), and cortisol (122,133). The conversion of progesterone to 16α -hydroxy-progesterone, 11β -hydroxy-androstenedione and cortisol has been observed in adrenal minces from twin female fetuses (122).

Bloch et al (134) isolated cortisol when progesterone was added as substrate to fetal adrenals in organ culture.

When progesterone was incubated with fetal adrenal slices, formation of cortisol, 11-deoxycortisol, androstenedione and 11β-hydroxy--androstenedione was demonstrated by Millner and Mills (135).

Studies with fetuses perfused with labelled progesterone indicate a high accumulation of radioactive material in the adrenals, testes and liver (131). Following the perfusion of midterm fetuses with progesterone, Bird et al (136) isolated 20α-dihydroprogesterone, 16α-hydroxyprogesterone, 17α-hydroxyprogesterone and cortisol from fetal adrenal; 16α-hydroxy--progesterone, 6β-hydroxyprogesterone and corticosterone from the perfusate; and mainly the reduced metabolites from the liver. Following the injection of ^{14}C-progesterone 'in situ' into the umbilical vein, besides ^{14}C-labelled 6β--hydroxyprogesterone, cortisol, 16α-hydroxyprogesterone and 17α-hydroxy--progesterone Solomon et al (10) were able to isolate the ester sulfates of cortisol, 11-dehydrocorticosterone, corticosterone and desoxycorticosterone from the adrenals. However, unlike the data obtained from 'in vitro' incubation studies, no conversion to C-19 steroids could be detected in the studies of perfusion experiments or injection of the precursor into the umblical vein. This would suggest that the ability of the intact fetal organism to remove the side chain of α,β-unsaturated, 3-ketosteroids is extremely limited (59).

Fetal metabolism of corticosteroids

Pasqualini et al (137) have shown the formation of aldosterone from the adrenals of previable fetuses following perfusion of corticosterone.

Bird et al (136,138) reported that many corticosteroids formed by

the fetal adrenals from progesterone are rapidly converted to their corresponding sulfates.

Fetal metabolism of dehydroisoandrosterone, androstenedione and testosterone

Colas et al (139) as well as Simmer et al (140) showed that there is a high concentration of dehydroisoandrosterone sulfate in umbilical arterial plasma at term, suggesting that major quantities of this compound are produced by the fetus. An analysis of report on the 'fetal metabolism of pregnenolone' (46,141,128) tend to indicate that the most likely precursor of the large quantities of dehydroisoandrosterone sulfate in umbilical arterial blood is pregnenolone of placental origin. However, Shimizu et al (142) have succeeded in demonstrating another pathway for the formation of dehydroisoandrosterone. Following the incubation of fetal adrenal slices with 20α-hydroxy-cholesterol, they were able to isolate 17α, 20α-dihydroxy--cholesterol and dehydroisoandrosterone. They suggested that the fetal adrenal is capable of converting cholesterol to C-19 steroids without the formation of a C-21 intermediary product. Whether this pathway is operative 'in vivo' remains to be established.

Bloch et al (143) reported that homogenates of fetal adrenals or testes can convert dehydroisoandrosterone to androstenedione. Archer et al (144) incubated minces of various fetal tissues and found androstenedione as a metabolite of dehydroisoandrosterone in the adrenals and testes. The formation of dehydroisoandrosterone sulfate and androstenedione from dehydro--isoandrosterone by the midterm fetal adrenal was also demonstrated by Shirley et al (145). The same steroids together with 11β-hydroxy-androstene--dione were also isolated following the incubation of adrenals from a newborn hydrocephalic infant (145).

However, in perfusions of previable fetuses, Bolté et al (146) indicated that radioactive dehydroisoandrosterone and dehydroisoandrosterone--sulfate were not converted to androstenedione or testosterone.

When previable fetuses were perfused with labelled dehydroisoandrosterone and dehydroisoandrosterone sulfate, both were exposed to extensive 16α-hydroxylation in the liver; was shown by Bolte et al (146). These authors indicated that the uptake of circulating dehydroisoandrosterone was very limited in all fetal tissues except in the liver (146). Moreover, the incubation of dehydroisoandrosterone with fetal liver homogenate yielded 16α-hydroxy-dehydroisoandrosterone (147). It has been assumed that 16β--hydroxylation of dehydroisoandrosterone sulfate takes place also in the liver (148). It has been shown that the 16-hydroxylation of C-21 precursors takes place in the fetal adrenals (10,141,123), as well as in the fetal liver (10); that of C-19 neutral precursors seems to take place mainly in the fetal liver and that of phenolic precursors in the maternal liver (146, 150,149).

Mancuso et al (87,88) have shown that the fetal liver can not aromatize β,γ-unsaturated steroids, such as dehydroisoandrosterone since it cannot convert such compounds to α,β-unsaturated, 3-ketosteroids which are obligatory intermediates in the process of aromatization. Whereas androstenedione and testosterone can be converted to estrogens by the liver of perfused fetus (88).

Following the perfusion of midterm fetuses with labelled androstene--dione and testosterone significant quantities of 11β-hydroxy-androstenedione were isolated from the adrenals and perfusates and some adrenosterone from the lungs (87).

Fetal tissues have been shown to conjugate β,γ-unsaturated

steroids into their 3-sulfates (151,152) as well as to 3β, 17-yl disulfates (153). Bolté et al (146) perfused the previable fetuses with dehydroisoandro--sterone and showed the extensive sulfurylation of this steroid.

Bertrand and Saez (154) reported that the concentrations of testosterone sulfate are much higher than that of testosterone in umbilical blood. Indeed, previable fetuses perfused with testosterone and androstene--dione converted these steroids into testosterone sulfate. The principal sites of this sulfurylation seems to be adrenals and the gastrointestinal tract (87). On the other hand little, if any, hydrolysis of testosterone sulfate has been shown in the tissues of perfused fetuses (78).

Fetal metabolism of estrogens

Estrone and estradiol are subjected to extensive interconversions in the fetal organisms (155-157). This reaction is most extensive in the liver (156), and is not influenced by conjugation with sulfuric (157,158) or glucuronic (159) acids.

Both 16α-and 16β-hydroxylated products (156,160,150,161) as well as 15-hydroxylated products (160) of estrogens have been reported in the fetus. The predominance of estriol over estrone and estradiol has been shown in the tissues of midterm fetuses, amniotic fluid (162), meconium (163) and urine of newborn infants (164,165).

Hormonal control of the fetal adrenal

The factors responsible for the control of adrenal growth and function in the fetus or newborn are not clearly established.

Lanman (166) suggested that ACTH from the fetal pituitary controls the growth of the 'fetal' zone. In the anencephalic fetus, the hypothalamus is invariably absent, although the anterior lobe of the pituitary is present (167). The adrenal cortex is very small in these fetuses. This is due mainly,

to a virtual absence of the 'fetal' zone (167-169). Since enlarged adrenals with prominant 'fetal' zones were found in surviving anencephalic infants given ACTH (166), ACTH has been implicated as the factor responsible for the growth of the 'fetal' zone (166). Taylor et al (170) detected ACTH in pituitary glands of 16-week-old fetuses. Berson and Yalow (171) reported that the mean concentration of this hormone in cord plasma was (161 pg/ml) three times greater than the value recorded for maternal plasma (56 pg/ml). Following the intraamniotic administration of ACTH to fetuses at mid--pregnancy, marked ultrastructural changes were observed both in the 'inner' zone and transitional area of the fetal adrenals by Johannisson (172). The findings suggested that this tropic hormone is capable of stimulating the fetal adrenal cortex.

Meyer (173), Kiyono (174) and Benirscke (175) noted that the fetal adrenal develops normally in anencephaly up to 20 weeks of gestation, which implies that some extra pituitary factor is involved in maintaining adrenal growth during early gestation. HCG has been suggested as a factor required for the development of the 'fetal'zone. The intraamniotic administ--ration of large doses of chorionic gonadotrophin at mid-pregnancy induced ultrastructural changes in the 'inner'zone of the fetal adrenal cortex, such as an increase in size and electron density of the mitochondria (172). Furthermore, when previable fetuses were perfused with a highly active antiserum (raised against chorionic gonadotrophin in rabbits) it induced specific changes in the ultrastructure of the fetal adrenals, which were highly suggestive of a decreased secretory activity (172).

Rotter (176) and Jones (177) had postulated that HCG was the fetal adrenotropic factor. However, opinion was held by Lanman and Dinerstein (178) as well as by Burner (179) that although HCG is produced in trophoblast,it tends to be secreted unidirectionally into the maternal circulation and is

not intended for use by the fetus. Studies conducted by Lauritzen in 1965 (180), have demonstrated the presence of HCG in the newborns during the first days of life. HCG is also excreted in the meconium and in the faeces during the first days of life (180-182) and is also found in the adrenals (1 to 2 I.U. per gram dry weight) (183). Measuring the HCG by ventral prostate test described by Greep et al (184), Lauritzen (180) showed that the concen--trations of HCG were significantly higher in the umbilical veins than in the arteries of newborn babies. When HCG was administered to the newborns, some 3-4% of the dose administered was excreted in the urine. This suggested a physiological role for HCG in the fetus. Geiger et al (185) in 1971, in the comparative radioimmunological determinations of HCG in the infant's and the mother's blood after delivery,have shown the blood concentrations of this hormone in agreement with the above authors. However, they failed to demons--trate a statistically significant difference between the HCG concentration in the umbilical vein and in the artery.

Lauritzen and Lehmann (186,181) demonstrated that the intra-muscular injection of 3,000-10,000 I.U. of HCG to newborns significantly increased the urinary excretion of dehydroisoandrosterone. This rise was even more pronounced in premature infants (181). These authors suggested that HCG is adrenocorticotropic hormone in the fetus, regulating the supply of fetal adrenal dehydroisoandrosterone as a precursor for the production of estrogens in the placenta.

Lauritzen et al (187) showed that the administration of cortico--trophin to newborns resulted in a markedly increased urinary elimination of cortisol and its metabolites, whereas, the increase in the urinary excretion of 3β-hydroxy- Δ^5-steroids was limited; suggesting that corticotrophin and chorionic gonadotrophin affect different enzymic processes in the newborn

adrenals. Ultrastructural studies conducted by Johannission (172) also suggest that the steroidogenetic activity of the human fetal adrenal, at least during the last part of gestation, is stimulated both by ACTH and HCG.

Brody and Carlstrom (188) have shown a relationship between HCG and the sex of the fetuses; mothers with male fetuses have a significantly lower level of serum HCG during the last trimester of pregnancy than those bearing female fetuses.

SECTION III.

Biosynthesis of estrogens in the feto-placental unit

It has been repeatedly demonstrated that integrated fetus and placenta are responsible for the elaboration of large amounts of estrogens during pregnancy. Cassmer (189) reported that interruption of the umbilical circulation, leaving the placenta'in situ', resulted in an immediate marked drop in estrogen concentration in the placenta as well as in the maternal urine. On the other hand these estrogen concentrations are not significantly reduced by the removal of either maternal ovary (190), adrenal (191) or pituitary (192) during the latter part of pregnancy. Whereas, urinary estrogen concentrations are low in pegnant women bearing anencephalic monsters (193,194) or in case of hydatiform mole (195). This is in keeping with the theory of Frandsen and Stakemann (196,194), who suggested that in normal pregnancy the fetal adrenals are producing large quantities of a precursor, which is converted to estrogens by the placenta. That this precursor could be dehydroisoandrosterone sulfate was indicated by Mancuso et al (197). Indeed, the administration of labelled dehydroisoandrosterone and/or its sulfate to pregnant women (198-201) led to the formation of

large amounts of estrogens, whereas, little if any, estrogens are formed from circulating dehydrisoandrosterone and its sulfate by the ovaries (202).

The placental aromatization of fetal precursors plays a paramount role in the formation of estrogens was demonstrated further following the injection of dehydroisoandrosterone into the intact umbilical (201,150) or uterine (82) circulation and particularly, following the 'in situ' perfusion of midterm placentas with androstenedione, testosterone, 19-nortestosterone, dehydroisoandrosterone and dehydroisoandrosterone sulfate. Perfusion with any of these precursors resulted in the formation of large quantities of estrone and estradiol-17β, but no estriol was detected (81,74,203,94,70,71). However, estriol was always isolated from the midterm placenta when dehydro--isoandrosterone or its sulfate was introduced into the feto-placental circulation directly (150) or via uterine artery (82). Estriol was also isolated from the placenta following the injection of androst-5-ene-3β, 16α, 17β-triol into the intact feto-placental circulation (204), or following 'in situ' placental perfusion with 16α-hydroxy-dehydroisoandroste--rone (70), or with 3β, 17β-dihydroxyandrost-5-en-16-one (71). The last mentioned perfusion also resulted in the synthesis of 16-oxo-estradiol and 16-epiestriol. In view of these data it is concluded that, the 16α-hydroxy--lation required for the formation of estriol takes place in the fetal and/or maternal organisms and not in the placenta (12).

During the first two months of pregnancy, the maternal ovary and in particular the corpus luteum is responsible for the production of estrogens. Initially, both estrogens and progesterone are necessary for the proper development and maintenance of the endometrium for successful implantation of the fertilized ovum. By the ninth week of pregnancy, steroid production by the ovary wanes (205,206); after this time ovariectomy can be carried out

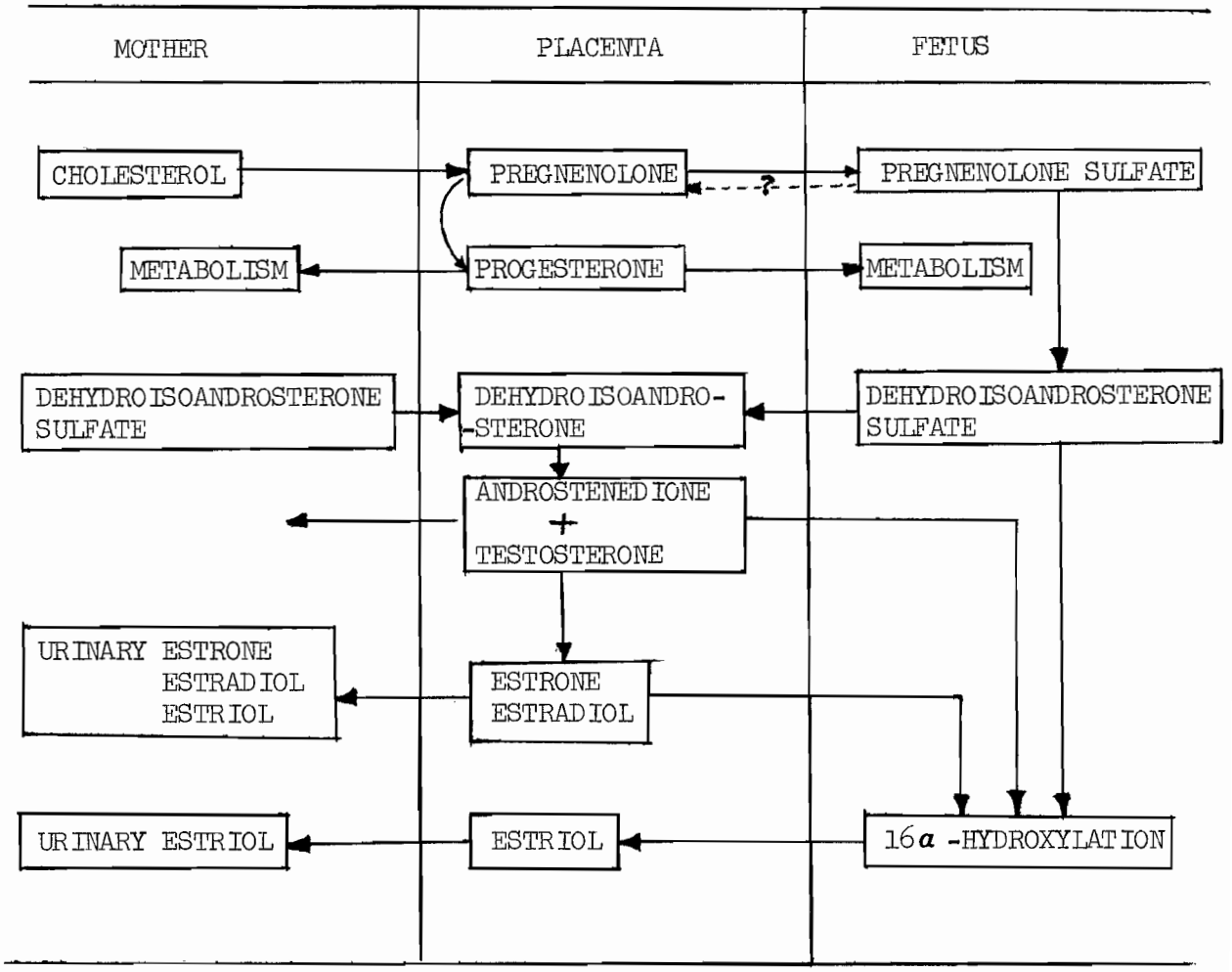
with much less risk of interruption of the pregnancy (207-210). Nevertheless estrogens and progesterone production continue to increase. From this time onward both the growing fetus and placenta are responsible for the production of most of the estrogens, and the placenta for that of progesterone.

Many attempts have been made to explain the highly increased amounts of estrogens during pregnancy. Investigators have sought an increased require--ment for estrogens,either to maintain the pregnancy or as a factor in the induction of parturition. Estrogens exert their major action on the uterus. During pregnancy the action of estrogens on the uterine muscle, i.e., on its growth, metabolism, and electrophysiological behaviour appears to be most important (103), Other investigators (211-213) have suggested that while estrogens are essential in early pregnancy for implantation of the fertilized egg, no clear necessity for their large production in late pregnancy has been demonstrated. Indeed, mothers excreting very low quantities of estrogens (e.g., in anencephaly, congenital adrenal hypoplasia, or congenital sulfatase deficiency) have infants which often survive to term and are of almost normal body weight (214-217). Similarly estrogens do not appear to be involved directly in the initiation of labour (218). Effects of estrogens on placental enzyme systems (219,220) and their mode of action has been studied extensively (221-223), yet the function of estrogens in pregnant women remains unknown.

Therefore a continuing and increasing level of estrogen production after the ninth week of pregnancy, depends primarily on an adequate and increasing supply of androgen sulfates, particularly of dehydroisoandrosterone sulfate and 16α-hydroxy-dehydroisoandrosterone sulfate, reaching the placenta from the fetus (224). The broad outline of the major pathways of estrogen biosynthesis at mid-pregnancy, when the feto-placental unit has assumed a dominant role in estrogen production is shown in Figure 1 (3).

FIGURE 1

INTEGRATED PATHWAY FOR THE BIOSYNTHESIS OF ESTROGENS IN THE FETO-PLACENTAL UNIT AT MID-PREGNANCY (3)



It seems that maternal cholesterol is a major source of pregnenolone in the placenta. Pregnenolone which reaches the placenta from both the maternal and fetal compartments, is extensively converted to progesterone by the placenta. This progesterone is secreted both to the maternal and fetal compartments, where it is further metabolized. Dehydroisoandrosterone sulfate formed in the fetal adrenal from placental pregnenolone is 16α-hydroxylated by the fetal liver (147,225,146). 16α-Hydroxy-dehydroisoandrosterone sulfate thus formed reaches the placenta and is aromatized to estriol (95,150,70), whereas dehydroisoandrosterone sulfate from the maternal adrenal and from the fetal adrenal serves as a precursor for the formation of estrone and estradiol in the placenta. The estrone and estradiol formed in the placenta can be trans--ported to the maternal circulation, where they are in part metabolized to estriol, or they can be transported to the fetus, where they are in small part 16α-hydroxylated. The major portions of the estrone and estradiol reaching the fetus are sulfurylated and it is as conjugates that they are transported back to the placenta and hydrolyzed. Then they reach the maternal circulation and contribute to maternal urinary estradiol and estriol (3).

It has been shown that ingestion of corticosteroids by the mother, reduces the concentration of dehydroisoandrosterone sulfate and 16α-hydroxy--dehydroisoandrosterone sulfate in cord blood (226). Urinary estrogen excretion is also suppressed by this treatment (227-231). This effect is interpreted as a consequence of the inhibition of ACTH secretion not only by the maternal but also by the fetal pituitary which results in a diminition of androgen secretion from the fetal and maternal adrenals. On the other hand ACTH injected into the mother increases the excretion of estriol (232,230) and to a lesser extent the excretion of estrone and estradiol-17β (233). Metyrapone, a drug which stimulates ACTH secretion by interference with cortisol biosynthesis (234) has been used

to examine the influence of ACTH on estrogen production. When pregnant women were treated with Metyrapone the excretion of estriol was increased signifi--cantly (230,235). Oakey and Heys (236,237) confirmed these findings in two pregnancies with hydrocephalic fetus as well as in four normal pregnancies. However, no increased response was detected in three pregnancies with an anencephalic fetus. Therefore it was concluded that an intact hypothalamic--pituitary—adrenal axis in the fetus was necessary to obtain a response.

In brief, it has been concluded by Oakey (224) that estrogens are synthesized in the placenta largely from androgen sulfates secreted by the 'fetal' zone of the fetal adrenal. Evidence has been presented that ACTH secreted by the fetal pituitary is the factor which stimulates androgen production by the fetal adrenal. In turn the secretion of ACTH is regulated by the concentration of cortisol in the fetus (224).

Indeed the fetal plasma has a lower capacity for cortisol binding than maternal plasma, as there are fewer binding sites available in cord plasma (238-241). This phenomenon in the fetus could be due to the fact that either there is a low concentration of cortisol binding globulin, or there is a high concentration of progesterone which competes with cortisol for the binding site in the fetal plasma (242-244). Maternal plasma with relatively lower concentration of progesterone, has much higher number of binding sites (238,239) available; with the result cortisol secreted by the fetus might pass over to the mother. A transfer of cortisol in the reverse (245,246) direction had been recognized, but occurs at delivery when under stress the maternal cortisol production rate increases (247) and large quantities of non-protein bound cortisol are available for diffusion from the mother to the fetus.

Transfer of cortisol from the fetal to the maternal circulation

results in an increased metabolic clearance of cortisol by the fetus, an increased ACTH secretion by fetal pituitary compensates for this increased cortisol clearance rate (224). In the adult, excessive ACTH secretion induces increased steroid production besides adrenal growth (248). An analogous response would be expected from the adrenal of the fetus also. However, from different data available Oakey (224) has concluded that the fetus synthesizes cortisol largely from placental progesterone in the 'fetal' zone of the adrenal. Since ACTH stimulates corticosteroid biosynthesis from cholesterol, but not from progesterone (249), no direct stimulation of cortisol production would be expected in the fetus. Increasing concentrations of ACTH would therefore provide the stimulus for an increased production of pregnenolone sulfate and dehydroisoandrosterone sulafte from cholesterol in the second half of pregnancy. As a consequence, there would be increased production of estrogens in the placenta (224). After birth, cortisol production must proceed in the 'definitive' zone from cholesterol as in the adult (250), since the supply of progesterone from the placenta is withdrawn and the 'fetal' zone degenerates.

However, this hypothesis leaves many questions unanswered. It is not known why 3β-hydroxysteroid dehydrogenase enzymes in the fetal adrenal remain inactive during gestation. Kinetic studies by Whitehouse and Vinson (251) strongly indicate a relative inactivity of at least one 3β-hydroxy--steroid dehydrogenase in the fetal adrenal during early gestation. Villee (252) has demonstrated inhibitory effect of progesterone on these enzymes 'in vitro' , which might suggest a possible explanation.

The hyperplastic 'fetal' zone degenerates after birth and involutes within about three months (253). The reason for this is not known, possibly as ACTH secretion falls after birth in response to a reduction in cortisol

clearance rate, the 'fetal' zone cannot be maintained and therefore degenerates. However, in the newborn infant, with congenital adrenal hyperplasia where plasma ACTH secretion is considered to be high and remains high without treatment (254), the 'fetal' zone degenerates (255). A reduction in plasma ACTH cannot therefore be the only factor responsible for the initiation of the 'fetal' zone degeneration (224).

Pauerstein and Solomon (256) have demonstrated a role for HCG in the control of adrenal androgen production in the adult. The influence of this hormone is removed at birth. Despite the findings of Lanman (166) that HCG did not stimulate adrenal growth in anencephalic infants, a function in the regulation of androgen production deserves consideration, specially in the period prior to the twentieth week of pregnancy.

It must be recalled that addition of HCG increased significantly the conversion of dehydroisoandrosterone, androstenedione and testosterone to estrone and estradiol by midterm or term placentas perfused 'in vitro' (109). This would suggest that HCG may regulate the extent of placental aromatization. Findings of Lauritzen et al (186,181) as well as of Johannisson (172) also strongly suggest that this hormone may be involved in the regulation of certain phases of the fetal steroidogenic processes.

While the mechanisms regulating the various phases of the steroid synthetic processes in pregnancy are put forward in this review, the extent to which such processes might be inter-related remain unresolved. This is not an easy task, and all the more so since we still possess very scarce information on this subject.

II . PURPOSE OF THE INVESTIGATION

There is good evidence that enzymatic systems in the placenta and in the fetal adrenals compliment each other during steroidogenesis, estrogens representing quantitatively the principal end products of such processes (1,2). It is also well established that dehydroisoandrosterone and dehydroisoandrosterone sulfate secreted by the fetal adrenals are precursors for synthesis of estrogens by the placenta (197-201,150,82). Besides progesterone and estrogens, the placenta is the site of production of HCG. Very little is known about the function of HCG in human, although its urinary titer is used for the diagnosis of pregnancy. 'In vitro' perfusion studies of term placentas showed that HCG significantly improves the yield of estrone and estradiol from C-19 neutral steroids (such as dehydroisoandrosterone, testosterone and androstenedione) (109). Furthermore, administration of HCG to newborns brought a significant increase in the urinary excretion of dehydroisoandrosterone (186,181). It has been suggested that HCG acts as an adrenocorticotrophic hormone in the fetus, regulating the supply of fetal adrenal dehydroisoandrosterone as a precursor for the production of estrogens by the placenta (180,181).

The present investigations were undertaken as an attempt to explain the steroidogenic role of HCG in the human term placenta and in the newborn adrenal and to explore the metabolism of C-19 and C-21 steroids by these tissues.

Since the availability of adrenal glands from infants who lived for less than 72 hours is extremely limited, adrenal glands from rats were also chosen to explore various enzymatic processes under the influence of HCG.

^{14}C-Labelled steroid precursors were incubated with different preparations of human placentas, newborn infant's adrenals and rat adrenals in the presence of a variety of co-factors, known to enhance certain aspects of steroidogenesis in these tissues. Emphasis was placed on the action of HCG on steroidogenesis.

We have also attempted to localize and study these enzymatic reactions at the subcellular level in the placental and in the adrenal tissues and the extent to which HCG affects such reactions.

III . MATERIALS AND REAGENTS

1. Buffer solutions

A Krebs-Ringer bicarbonate buffer solution (257) pH 7.4 containing 200 mg % glucose (KRBG) was the medium used for all the incubations. The buffer was freshly prepared for each set of experiments from mother solutions and gassed with 95% O_2 - 5% CO_2 for approximately five minutes before use.

2. Co-factors and enzymes

The following were purchased from Sigma Chemical Co., St.Louis, Mo., U.S.A.

NADP (Nicotinamide adenine dinucleolide phosphate monosodium salt); G-6-P (Glucose-6-phosphate disodium salt); 3′, 5′-AMP (Adenosine 3′, 5′--cyclic monophosphate) 3′, 5′-AMP dibutyryl (2′-dibutyryl 3′,5′-cyclic adenosine monophosphate sodium), G-6-P-dehydrogenase (Glucose-6-Phosphate dehydrogenase, crystalline suspension in 2.6 M ammonium sulfate solution, 0.9 mg protein per ml).

HCG (Human chorionic gonadotrophin) was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

3. Non-radioactive steroids

Commercially available steroids were purchased from Steraloids Inc., or Merck. They were used as internal standards or for the dilution of the respective radioactive steroids employed as precursors in incubations, or as carriers for crystallization of the corresponding radioactive compounds.

4. Radioactive steroids

All radioactive steroids were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. ^{14}C-labelled steroids were used as precursors in incubations and ^{3}H-labelled steroids as indicators for correction of losses. The following compounds were used:

	Specific activity
4-^{14}C-Pregnenolone	52.8 mc/mmol
4-^{14}C-Progesterone	50.0 mc/mmol
4-^{14}C-11-Deoxycorticosterone	54.3 mc/mmol
4-^{14}C-Dehydroepiandrosterone	58.8 mc/mmol
4-^{14}C-Testosterone	58.8 mc/mmol
4-^{14}C-Androstenedione	58.8 mc/mmol
7-^{3}H-Pregnenolone	25.0 c/mmol
1,2-^{3}H-Progesterone	40.0 c/mmol
1,2-^{3}H-11-Deoxycorticosterone	10.0 c/mmol
1,2-^{3}H-Corticosterone	20.0 c/mmol
1,2-^{3}H-Aldosterone	20.0 c/mmol
7-^{3}H-Dehydroepiandrosterone	12.9 c/mmol
1,2-^{3}H-Testosterone	45.0 c/mmol
1,2-^{3}H-Androstenedione	40.0 c/mmol
6,7-^{3}H-Estrone	40.0 c/mmol
6,7-^{3}H-Estradiol-17β	40.0 c/mmol

The chromatographic homogeneity of these labelled steroids was verified prior to their use.

5. Radioactive acetic anhydride

These were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. ^{3}H-acetic anhydride (50 mc/mmol or 100 mc/mmol) was used at a concen--tration of 12% in benzene. ^{14}C-acetic anhydride (10 mc/mmol) was used at a concentration of 20% in benzene. The specific activity of these reagents was further verified as described by Kliman and Peterson (258).

6. Reagents

All reagents used for extraction and chromatography were purified and redistilled according to the procedures recommended by Bush (259).

Acetic acid, acetic anhydride (non-radioactive), benzene, and pyridine were distilled according to Kliman and Peterson (258) and stored at 5°C. in a vacuum desiccator over a layer of calcium chloride.

7. Scintillation fluid for counting

Four grams of 2,5-diphenyloxazole and 0.1 gram of 1,4-bis-2 (5-phenyloxazolyl) benzene (Packard Instrument Co., La Grange, Ill., U.S.A.) were dissolved in one litre of redistilled toluene.

8. Glassware and vials

All glassware used were clean and free from radioactivity. Vials (Potassium free No-Sol-VIT glass) in which aliquots of radioactivity were taken for counting, were used only once.

IV . METHODS

A) General

1. Chromatography systems

Mostly thin-layer, but some paper chromatography systems have been used for the separation and purification of steroids.

a) Thin-layer chromatography

Ready made thin-layer chromatography glass plates (20 X 20 cm) were purchased from Brinkmann Instruments (Canada) Ltd.These plates were evenly coated with 0.25 mm layer of Silica gel F-254 containing 1% of phosphor to facilitate the detection of Δ^4-3-Keto steroids under ultra--violet light (240 mμ). In order to have reproducible running rates of the different steroids, the walls of the chromatographic tanks were lined with chromatography paper (Whatman no. 3) saturated with the solvent system. Besides the steroid extracts from the experiments, appropriate authentic steroid standards of known R_f values were applied on each thin-layer chromatography plate. The plates were developed at room temperature and the time of development varied from 90 to 120 minutes, depending upon the system. Sometimes,after the plate was allowed to dry for five minutes, it was rerun for the second or third time in the same system to achieve a better resolution of steroids. A list of TLC systems used is indicated in Table I. These different systems will henceforth be referred to by the Arabic number assigned to each in this table.

b) Paper chromatography

Whatman no. 1 chromatography paper was used unwashed after scanning

TABLE I

THIN LAYER CHROMATOGRAPHIC SOLVENT SYSTEMS

Systems	Composition		Reference
T L C- 1	Chloroform : Ethanol	(19:1)	
T L C- 2	Ethyl acetate : n-Hexane	(1:3)	
T L C- 3	Cyclohexane : Ethyl acetate	(1:1)	(260)
T L C- 4	Benzene : n-Butyl acetate	(19:1)	
T L C- 5	Ethyl acetate : n-Hexane	(1:1)	(261)
T L C- 6	Chloroform : Acetone	(9:1)	(261)
T L C- 7	Benzene : Acetone	(4:1)	(262)
T L C- 8	Methylene chloride : Diethyl ether	(5:2)	(263)
T L C- 9	Methylene chloride : Acetone	(4:1)	(264)

under ultraviolet light (250 mμ) to ascertain that it was devoid of U.V. absorbing or fluorescent impurities. The paper was cut into strips (43 cm long, 2 cm wide, 1 cm apart) attached to a common head 13 cm long. The line of application was 2 cm from the common head. The systems and their solvent composition are listed in Table II.

2. Detection of steroids on chromatograms

a) Radioactive materials

i) Radiochromatogram scanning

Dry paper strips were scanned in a radiochromatogram scanner (Packard Model 7200). The sensitivity was adjusted according to the expected amount of radioactivity in the steroid applied. This method was used for the purification of certain ^{3}H-steroid standards.

ii) Radioautography

Radioactive (^{14}C) products on thin-layer plates were located with x-ray films (cronex 2 DC Medical x-ray film, DU point). The plate was covered with a standard film in a x-ray cassette for a length of time, varying from 1 to 3 days, depending on the quantity of radioactivity expected in the conversion products. The films were developed by standard x-ray film developing technique.

b) Non-radioactive materials

i) α, β-unsaturated steroids

The localization of radioactive α, β-unsaturated compounds was facilitated by the addition of 10 to 20 μg of the respective non-radioactive internal pilots whenever circumstances allowed. The α, β-unsaturated steroids isolated on thin-layer or paper chromatograms were detected by ultraviolet light (mineralight UVS11)

TABLE II

PAPER CHROMATOGRAPHIC SOLVENT SYSTEMS

Systems	Composition	Reference
	Mobile phase [A] / Stationary phase	
Bush A	Benzene / Methanol : Water (100 / 50 : 50)	(265)
E_2 B	Isc-Octane / t-Butanol : Water (100 / 50 : 90)	(266)
	[B] Isopropyl ether : n-Hexane :	
B5-10	t-Butanol : Conc. NH_4OH : Water (5:2:3:1:9)	(267)

A. These were used for purification of authentic tritiated steroid alcohols.

B. This was used for the separation of steroid sulfates.

ii) Δ^5,3β-OH-steroids and C-18, $\Delta^{1,3,5}$,3β-OH-steroids

These compounds were located on paper or thin-layer chromatograms by spraying with a 10% solution of phosphomolybdic acid in absolute ethanol (268). The chromatograms were subsequently heated for 5 to 10 minutes at 80-90°C. The steroid appeared as dark blue spots against a yellow back--ground.

The above two techniques of detection were used in order to match the position of ^{14}C-labelled metabolites with known authentic radioinert standards, used either as carriers or as reference compounds.

3. Elution of steroids from chromatograms

a) Elution from thin-layer plates

The areas of silica gel containing steroid spots were extracted from the plate with a vacuum extractor. The device used was as follows: The wider end of a disposable Pasteur pipette (length $5\frac{3}{4}$", Fisher Co., Montreal.) was packed with 5 to 6 cotton pellets (no. 2, Denco, Montreal.). This end was connected to a vacuum pipe. Silica gel areas were eluted from the plate, via the thinner end of the Pasteur pipette, with the aid of vacuum. As a result, the silica gel collects in the Pasteur pipette just below the cotton pellets. With the aid of a syringe 3 times 2 ml of ethanol were added through the thinner end of Pasteur pipette to elute the steroids from the silica gel. The eluate was evaporated to dryness under a stream of dry air at 50°C.

b) Elution from paper

Areas on the strips containing the steroids were cut into very small pieces and left overnight in a flask containing 25 ml ethanol. The following day, ethanol was filtered, the pieces of paper were washed three times with 10 ml of ethanol and the pooled solvents were evaporated to

dryness under reduced pressure at 40°C.

4. Identification of steroids

General consideration

Studies conducted with ^{14}C-precursors under a variety of experi--mental conditions with either placenta or adrenals (from newborn babies or hooded male rats) yielded metabolites with identical chromatographic mobility with the internal or external pilot in two to three thin-layer chromatographic systems. The individual characterization of these metabolites with identical behaviour seemed an excessive and impractical task. Therefore the final and most elaborate steps of identification were carried out in pools of the analogous materials. It should be noticed however, that this mixing into pools was done only after the isopolarity of each metabolite in question was reasonably well established with the respective authentic steroid. A consider--ation of the individual steps comprised in the identification of steroid metabolites follows:

Step I. Isopolarity with cold carrier

The isopolarity of ^{14}C-labelled material and the carrier was established contrasting the dark zones of radioactivity in the radioautograms with purple spots under ultra violet light absorbing at 240 mμ (for steroids with a Δ^4,3-Keto structure). In the case of pregnenolone, dehydroisoandro--sterone, or estrogens spot tests with 10% solution of phosphomolybdic acid in ethanol were carried out on TLC plates or paper strips running simultan--eously with ^{14}C-labelled experimental substances.

This step was common to all steroid metabolites since it entails their isolation from the initial TLC system.

STEP II. Constant ^{3}H: ^{14}C ratios

Since before extraction of the incubation mixtures, known amounts of tritiated steroids were added in most cases to corresponding ^{14}C-metabolites in the incubation mixture, after these metabolites were initially separated from the crude extract, they were individually purified in 2 to 3 TLC systems. After each chromatography, aliquots were taken and ^{3}H : ^{14}C ratios were established. In the same way when the extracts were directly acetylated with ^{3}H-acetic anhydride, the constant ^{3}H : ^{14}C ratios of the resultant acetates were reached after several thin-layer chromatographies.

Step III. Formation of derivatives

At this stage, some of the steroids were subjected to further analysis by the formation of a derivative, which depended on the nature of the steroid under study. The derivatives of metabolites were formed by adopting the following procedures:

a) Acetylation of steroid compounds

i) With non-radioactive acetic anhydride

The dry steroid residue was dissolved in 0.30 ml dry pyridine and 0.15 ml of acetic anhydride was added. After standing overnight at room--temperature,the acetylation was stopped with ethanol. The excess reagent was dried under a stream of air. The acetylated extract was directly applied on TLC plate.

ii) With radioactive (^{3}H) acetic anhydride

This method was followed for the formation of derivatives of steroids, such as 19-OH-androstenedione, 19-OH-testosterone and 16α-OH--androstenedione. As these ^{3}H-labelled compounds are not commercially available. The ^{14}C-labelled 19-OH-androstenedione, 19-OH-testosterone or

16α-OH-androstenedione obtained from the experimental incubation were acetylated with ^{3}H-acetic anhydride in order to form resultant double labelled (^{3}H : ^{14}C) mono or diacetate.

The following method was used: Steroids were concentrated at the tip of an acetylation tube, which was allowed to get thoroughly dry for at least four hours in a vacuum desiccator. By means of pipettes flamed immediately before use and fitted in an "ultra-micro pipet-filter", 0.025 ml of pyridine and 0.03 ml of ^{3}H-acetic anhydride were added to the tube, which was stoppered immediately, and gently shaken to allow complete mixing of materials at the tip. The mixture was incubated for 48 hours in a constant temperature incubator at 37^{o}C.

The extraction was carried out according to the method of Kliman and Peterson (258). By subsequent chromatography of the resultant acetate ^{3}H : ^{14}C ratios could be establishedfor the compound.

b) Oxidation of the 11β-hydroxyl group of the steroid with chromium trioxide

Oxidation of the 11β-hydroxyl group of the steroid was carried out essentially as described by Kliman and Peterson (258). The product, thoroughly dried in a 4 ml capacity pyrex tube, was oxidized with 0.1 ml of 0.5% chromium trioxide in 95% acetic acid.

Step IV. Microcrystallization of steroids

This was a final step in the characterization of steroids. The microcrystallization method of Axelrod et al (269) was used. The radioactive metabolites or their derivatives were crystallized to constant specific activity. When possible, constant ^{3}H : ^{14}C ratios were also established. Crystallization of the ^{14}C-metabolites were carried out only after the identity of each steroid was established by the previous criteria (see step I-III).

The carrier compound to be added was previously crystallized three times and the crystals thus obtained were dried in a vacuum desiccator for 24 hours, before its addition to the ^{3}H : ^{14}C steroid. About 20 to 25 mg of the carrier steroid were mixed with the corresponding radioactive steroid in a pre-weighed tube.

Based on the results of Axelrod et al (269), acetone and pentane or ethanol and pentane were used as the only pair of solvents for the micro--crystallization. The mixture (i.e., carrier+unknown) was brought into a solution in about 1 ml of boiling acetone or ethanol, depending on the size and solubility of the sample. While boiling, the solution was constantly agitated with a glass rod to prevent boiling over, or bumping. The volume was reduced to about half while the sample was still in solution, it was removed from the water bath and pentane was added; a few drops at a time with agitation, until a cloud began to appear. As the mixture cooled, the cloud precipitated into crystals. When an adequate amount of crystals were formed, the tube was centrifuged for 3 to 5 minutes. The mother liquor was carefully decanted into a pre-weighed tube. Pentane : methanol (20 : 1) was added to the crystals to help removing any remaining mother liquor. The tube was again centrifuged and the supernatant added to the first mother liquor. The crystals and the mother liquor were separately brought to dryness overnight under vacuum. Weights were taken and the residues, both in crystals and the mother liquor tubes, were brought into solution with ethanol. According to the amount of steroid present, an appropriate aliquot was taken from each sample into a counting vial. The crystals in solution were again dried and subjected to further crystallization. Usually three to four successive crystallizations were carried out. Each time weights in mg and total number of d.p.m. were established in crystals and mother liquors. The specific activity

of each crystallization was calculated as d.p.m./mg.

5. Assay of radioactivity

Assays of radioactivity were carried out in Packard Tricarb liquid scintillation spectrometer, model 4322, set at a single voltage (2700 Volts). Aliquots ranging from 1/100 to 1/2 of the total sample were used for the radioactive assays. Aliquots were pipetted out in the counting vials, dried and dissolved in 15 ml of the scintillating fluid. Since most of the samples contained ^{3}H and ^{14}C, simultaneous counting of ^{3}H and ^{14}C was carried using the discriminator ratio method of Okita (270), as modified by Stachenko et al (271). Each sample was counted for sufficient time (20 minutes) so that a standard error of the mean of less than 2% was achieved in both channels. Efficiencies for ^{14}C and ^{3}H under dual label conditions were 57% and 17% respectively.

No correction for quenching was necessary, since the trial with internal standards of ^{3}H or ^{14}C-toluene indicated that the samples were not quenched. Background counts were not more than 10 c.p.m. in the ^{3}H channel and 14 c.p.m. in the ^{14}C channel.

Under the counting conditions employed the ^{3}H-c.p.m. appearing in the ^{14}C channel were negligible (0.2%), that of ^{14}C-c.p.m. in the ^{3}H channel amounted to approximately 10% and were corrected for, by counting pure ^{14}C-standard with each batch of assays.

B) Experimental

1. Tissue preparation

a) Preparation of tissue from human placenta

Human term placentas, obtained immediately after normal delivery

from St.Luc Hospital in Montreal, were processed in cold and dissected free of fetal membranes. The placental tissue was teased free of large blood vessels and washed several times with normal saline. Three different kinds of preparations were used for incubations.

i) Placental minces

With the help of small scissors, placental tissue was cut into minces. The minces were kept in ice cold KRBG buffer. The buffer solution was decanted and the minces were rinsed twice with 5 ml of fresh ice cold buffer. The minces were gently blotted between layers of filter paper and weighed in aliquots of 1 gm. These minces were now ready for preincubations

ii) Placental homogenates

Homogenates were prepared from placental minces after they were subjected to one hour of preincubation period. The preincubated minces were transferred to a pestle homogenizer. The homogenates were prepared in cold ($4^{\circ}C$), using KRBG buffer as a homogenizing media. The homogenates for each incubation contained 1 gm of placental tissue per 6 ml of KRBG, unless otherwise mentioned.

iii) Subcellular fractions

The homogenates of placental minces were prepared in ice cold 0.25 M sucrose solution and transferred to the cup of a Virtis homogenizer surrounded by ice. The tissue was homogenized twice at high speed for 45 seconds with a one minute halt between. 26 gm of tissue were homogenized in total volume of 86 ml of 0.25 M sucrose. Thus 3.3 ml of sucrose contained 1 gm of placental mince. 20 ml of the homogenate were kept aside in cold ice till the time of incubations, to carry out the studies with homogenates as such. The remainder 66 ml were subjected to standard differential

centrifugation procedure to separate the various organelles. The homogenate was first centrifuged at 750 X g for 10 minutes at 4°C (Sorvall centrifuge). The pellet thus obtained was labelled as 'nuclei' and was discarded. The 750 X g supernatant was centrifuged for 10 minutes at 12,000 X g, (Spinco- - ultracentrifuge) the pellet obtained was re-suspended in 2 ml of 0.25 M sucrose and recentrifuged for the second time at 12,000 X g. The pellet obtained after the second centrifugation was called the 'mitochondrial pellet'. The supernatant of 12,000 X g and the washing at 12,000 X g were pooled and centrifuged at 105,000 X g for 60 minutes. The sediment was designated as the 'microsomal pellet' and the 105,000 X g supernatant was called 'supernatant'.

The organelles were resuspended in the homogenizing medium (0.25 M sucrose) in such a way, that when each sample incubated would be derived from 1 gm of placental tissue, and would be in a volume of 3.3 ml of 0.25 M sucrose. This provided comparable conditions with the whole homogenate, where each gram of tissue was suspended in 3.3 ml of 0.25 M sucrose.

In most of our studies with placenta, since the total incubation volume of the buffer (KRBG) in each incubation flask was 6 ml , to have the comparable conditions here, 2.7 ml of KRBG were added to each incubation flask already containing 3.3 ml of sucrose. These subcellular fractions were ready for incubations.

b) Preparation of tissue from human newborn adrenal

Adrenal glands were obtained within 4 to 6 hours of the death of human premature or full term infants at autopsy, who lived for less than 72 hours. Death was attributed to prematurity and/or to the respiratory dysfunction or to intestinal disorder. Permission for obtaining the glands was granted by the Pathology Dept., Montreal Children's Hospital. The glands

were freed of peripheral fat and processed in 0.9% cold saline. Only one kind of tissue preparation was used for the incubation here.

i) Adrenal minces

With the help of small scissors,minces were prepared from human adrenal glands and were washed two times with 5 ml of fresh KRBG, after they were gently blotted with filter paper, they were weighed out in aliquots of 180 to 400 mg , depending on the type of experiment and were placed in a vial, containing 5 ml of KRBG. These were ready to be preincubated.

c) Preparation of tissue from rats

Adrenal glands from male hooded rats (120 to 130 gm) were used for incubations. These glands were cleaned free of surrounding fat, and were processed in cold physiological saline solution. Two kinds of tissue preparations were used for incubations from this source.

i) Adrenal bisects

With the aid of a scalpel, each adrenal gland was cut into two equal halves. Aliquots of 228 mg of adrenal bisects were weighed, transferred to vials, rinsed two times with 5 ml of KRBG and were ready for preincubation.

ii) Subcellular fractions

The homogenizing media used for the separation of 'mitochondrial' and 'microsomal' fractions from the rat adrenals was 0.44 M solution of sucrose as has been described by Dounce et al (272). Each adrenal was separated into two parts, the 'glomerulosa' part (capsule-Glomerulosa) and a 'fasciculata' part(Fasciculata-Reticularismedulla) by a method reported by Giroud et al (273). The homogenates of 'fasciculata' (from 56 glands) and 'glomerulosa' (from 50 glands) were prepared in total volume of 15 ml. sucrose 0.44 M. The homogenates of whole glands (from 3 glands) were prepared in total volume of 2 ml sucrose 0.44 M.

The homogenates of the 'fasciculata' and the 'glomerulosa' were subjected to centrifugation in exactly the same way as described for the placenta. However, after obtaining the 12,000 X g 'mitochondrial pellet', no further centrifugation was carried out. The 12,000 X g supernatant was designated as the 'microsomal fraction'.

The 'mitochondrial pellet' was resuspended in the 0.44 M sucrose in such a way that each incubation of 'fasciculata' contained 0.36 gland equivalent of rat adrenal in 0.1 ml sucrose; each incubation with 'glomerulosa' contained 1.6 glands equivalent in 0.1 ml of sucrose. The incubations of whole glands were carried out with 0.36 gland equivalent.

It has been demonstrated in our laboratory (274) that enzymatic activities in the 'fasciculata' and in whole glands are significantly higher than those observed in the 'glomerulosa' when calculated per gland, or per weight. In order to have comparable enzymatic activities in the 'glomerulosa', in the 'fasciculata' and in whole glands, a greater amount of 'glomerulosa' had to be used per incubation.

2. Preincubation of tissue

Placental minces (used for incubations or for preparation of homogenates), adrenal minces (from newborn babies), or adrenal bisects (from rats) were always subjected to one hour preincubation period. The minces or bisects were placed in vial containing 5 ml of KRBG. The vials were preincubated at 37°C in a Dubnoff incubator under 95% O_2-5% CO_2 for one hour. The shaking rate was kept constant at 60 cycles per minute.

3. Incubations

At the end of the one hour incubation, the preincubated minces

(from placenta or newborn adrenals),or bisects(from rat adrenals) were tra--nsferred to another set of vials. Homogenates from placentas were prepared at this stage and transferred to vials. The incubation with minces, bisects, or homogenates were then carried out in vials containing known amounts of ^{14}C-labelled steroid precursors. Where indicated, freshly prepared solutions of additives were quickly pipetted into the experimental vials and volumes ad--justed to 3 ml or 6 ml according to the set up of the experiment and will be referred at appropriate places in'results'.

The vials were carefully swirled and put back into the metabolic shaker to be incubated for a subsequent two hour period (unless otherwise stated) under 95% O_2-5% CO_2.

4. Preliminary before extraction

In the case of studies with minces (either from placenta or from newborn adrenals), or bisects (from rat adrenals), the tissue was separated from the corresponding medium at the end of final incubation period. The minces or bisects were washed twice with 2 ml of KRBG and washings pooled with the original incubation medium in vial. The tissues remaining in the vials were homogenized. 10 ml of acetone were added to the homogenates as well as to the vial containing corresponding medium plus washings to stop the enzymatic reaction.

In the experiments where studies were carried out with the placental homogenates, or subcellular fractions, at the end of incubation period 10 ml of acetone were added to the vials containing homogenates or subcellular fractions.

All the samples were left overnight in cold at $4^{o}C$ during which period proteins were precipitated. The precipitate was filtered off and

washed three times with 3 ml of fresh acetone. The acetone was evaporated under a stream of air leaving behind the aqueous fraction. These aqueous fractions were now ready for extraction and further processing, depending on the parameter(s) studied i.e., %-conversion and/or the endogenous production. The aqueous fractions were either processed as described in part I (when only %-conversions from the added precursors were studied), or the aqueous fractions were divided into 2 parts and processed as outlined in Figure 3, (when %-conversions as well as endogenous productions of steroids were studied).

5. Extractions

Our preliminary method of extraction involved the separation of androgen-corticosteroids from estrogen fraction, as commonly reported in the literature. The C-19 and C-21 steroid alcohols were first extracted from the aqueous fraction twice, with equal volumes of methylene chloride. The Methylene chloride extract was washed twice, with 2 ml of 1 N NaOH. This was done, to bring certain percent of estrogen which are soluble in methylene chloride, into NaOH. The alkaline wash was pooled into the aqueous fraction which was left from initial methylene chloride extraction. The pH of this combined pool was adjusted to 9-9.5. Estrogens were extracted from this pool twice, with equal volumes of diethyl ether. Both the neutral fraction and phenolic fractions were washed with water up to neutral pH. The extracts were evaporated under the stream of air.

However, in spite of all the efforts, when used with ^{14}C-steroid standards, this method was not found very satisfactory, as 10-20 percent of estrogens were always detected into corticosteroids-androgen fraction and vice versa. We decided to seek for a procedure whereby a good separation between the estrogens, androgens and corticosteroids could be obtained. This,

we were able to achieve, as described below by chromatography (using TLC systems) of the crude extracts which contained the mixture of estrogens and androgens or androgens and corticosteroids.

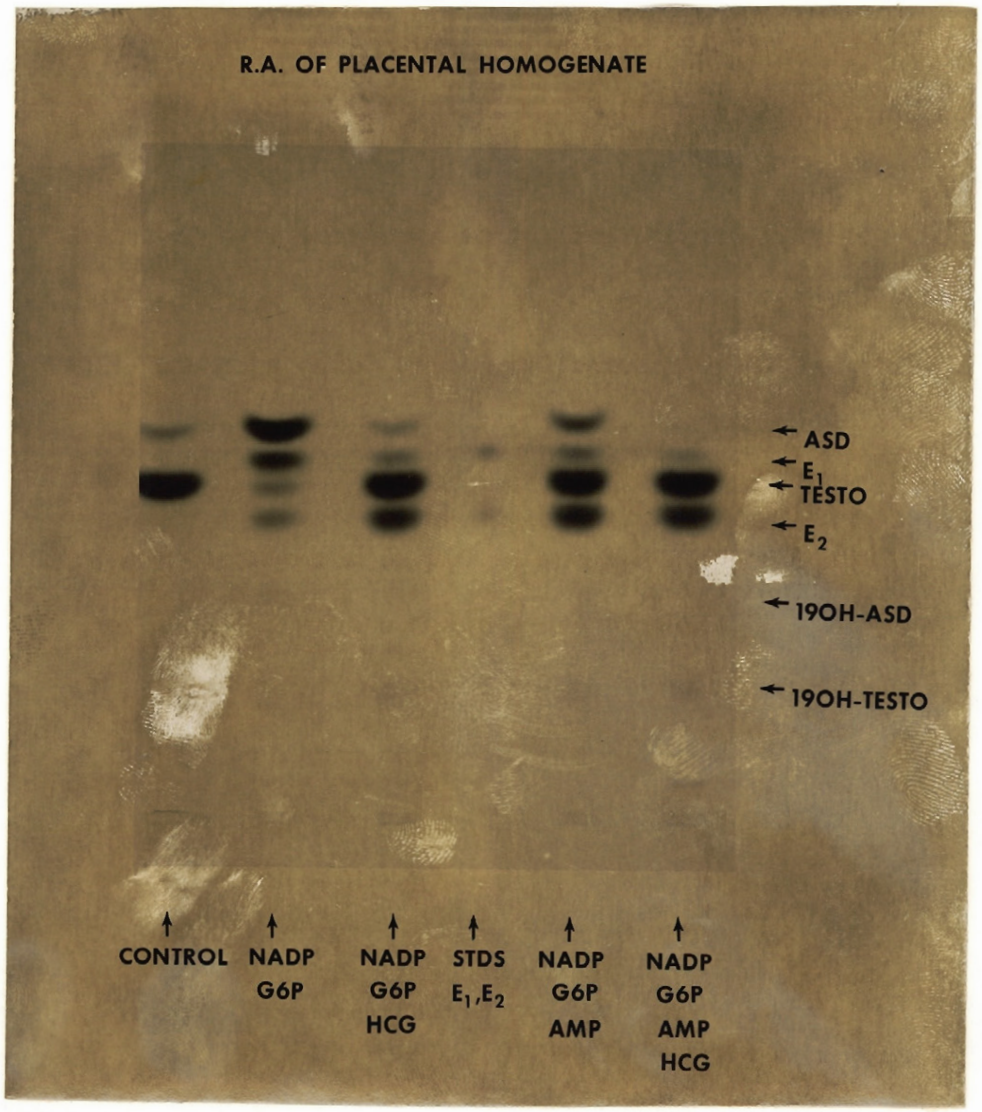
Each aqueous fraction or an aliquot of the aqueous fraction (from minces, homogenates or subcellular fractions) was extracted twice with equal volumes of methylene chloride and twice with equal volumes of diethyl ether, the methylene chloride and diethyl ether extracts were pooled and then dehydrated over anhydrous sodium sulfate and filtered. The sodium sulfate was washed three times with 3 ml of methylene chloride: diethyl ether (1 : 1) and the washings added to the filtrate. The total pooled extract was evaporated to dryness. The steroids in the different extracts were separated by TLC using various solvent systems. Figure 2 represents a photograph of a radioautogram on TLC I. One can see that, when a crude extract of placental homogenate was applied on this TLC system, there was a fairly good separation of various steroid metabolites.

To check the losses occuring during the whole experimental procedure, studies were conducted with known amounts of various ^{14}C and ^{3}H steroid standards, which were subjected to extraction and chromatography in exactly the same way as described for experimental incubations. In all instances, the counts recovered after extraction and first chromatography were 75-85% of the total counts added.

In the studies with newborn adrenals, after the extraction with methylene chloride and diethyl ether, the residual water was extracted twice with equal volumes of n-butanol. This was done in order to look for the possible sulfurylation of steroids in the baby's adrenal.

FIGURE 2

METABOLISM OF 4-^{14}C-TESTOSTERONE BY PLACENTAL HOMOGENATES IN THE PRESENCE OF VARIOUS CO-FACTORS.



This is an illustration of a photograph of a radioautogram (R.A) on TLC 1. This figure shows the typical conversion products from 4-^{14}C-testosterone to different steroids isolated from placental preparations under the conditions of incubations described in methods.

6. Processing of the incubation extracts

This method provides insight into the metabolic processes under--gone by endogenous precursors, the nature of which is either not reflected, or blurred, when the metabolic products of a labelled precursor are only assessed in terms of yield of radioactivity (275). It allows the simultaneous calculation of the isotopic content and of the mass of the steroid metabolites produced during the incubations with a radioactive precursor. The complete scheme is given on Figure 3.

Part I. Determination of the % conversions and exogenous productions (nmol) of steroid metabolites

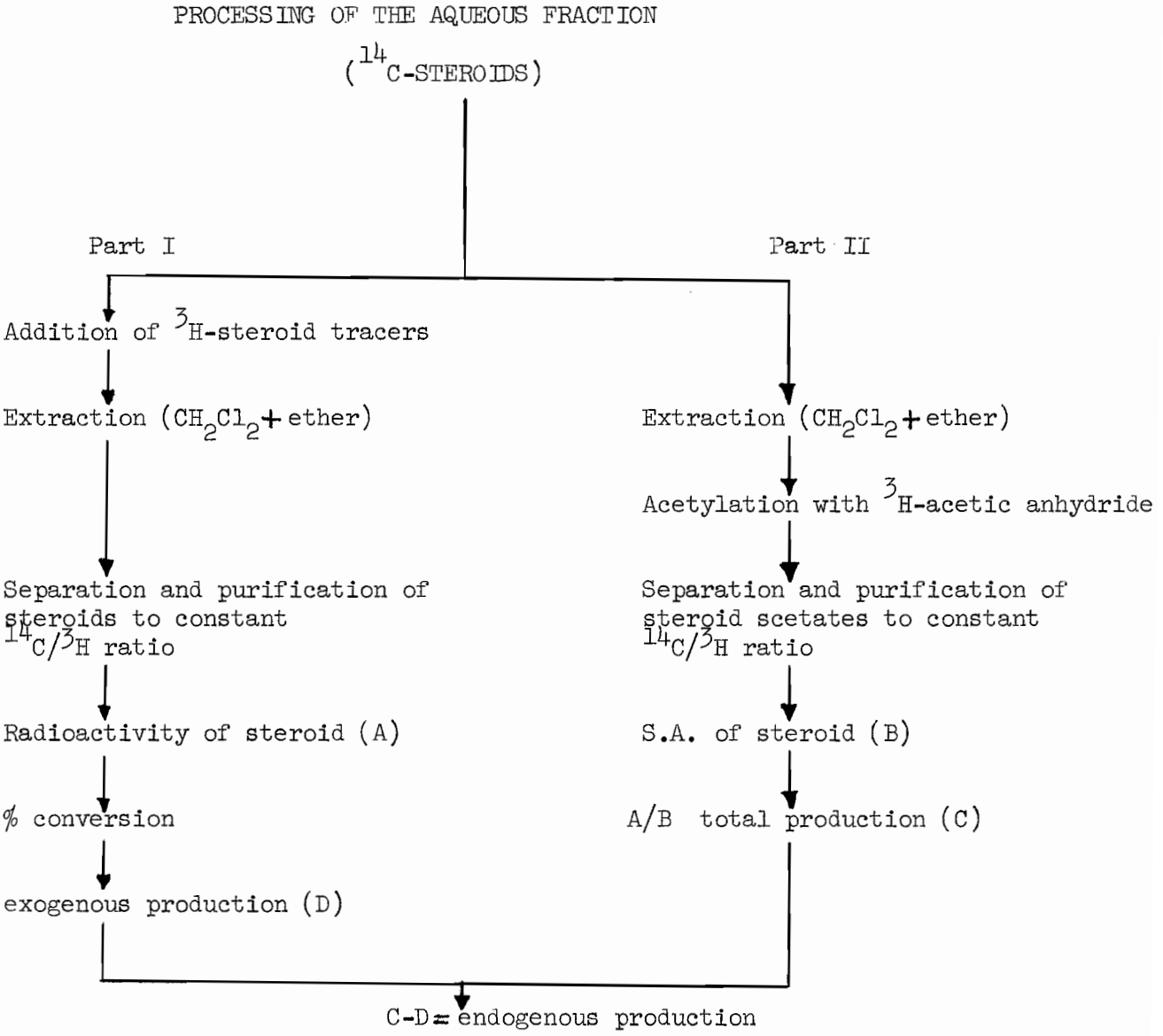
This part was either a known aliquot of the aqueous fraction or the total fraction and was processed in order to obtain the percent conversions of various metabolites from the ^{14}C-precursor. To this part non-radioactive internal standards (approximately 20 μg each), as well as corresponding tritiated steroid tracers of known and appropriate radioactivity were added. This was done to correct the losses of the corresponding ^{14}C--conversion products during purification processes. After the extraction as described above, the steroids were separated on TLC 1. Individual steroids were further purified by TLC in various chromatographic systems to a constant ^{14}C : ^{3}H ratios. When possible, acetylation and purification of steroid acetates was carried out to constant ^{14}C : ^{3}H ratios. This ratio was applied to the calculation of the %-conversion of the added labelled substrate, as well as to the yield (nmol) of the exogenous radioactive products.

Determination of the %-conversion and production (nmol) from an exogenous radioactive precursor for any steroid:

Taking estrone as a conversion product, let

R_1 = constant $^{14}C/^{3}H$ ratio of isolated ^{14}C-labelled estrone to ^{3}H-labelled estrone indicator.

FIGURE 3


PROCESSING OF THE AQUEOUS FRACTION
(^{14}C-STEROIDS)
Part I
Part II
Addition of ^{3}H-steroid tracers
Extraction (CH_2Cl_2 + ether)
Extraction (CH_2Cl_2 + ether)
Acetylation with ^{3}H-acetic anhydride
Separation and purification of steroids to constant ^{14}C/^{3}H ratio
Separation and purification of steroid scetates to constant ^{14}C/^{3}H ratio
Radioactivity of steroid (A)
S.A. of steroid (B)
% conversion
A/B total production (C)
exogenous production (D)
C-D = endogenous production

D_i = radioactivity (d.p.m.) of ^{3}H-estrone added to part I.

D_p = radioactivity (d.p.m.) of added ^{14}C-labelled steroid precursor.

n = fraction of total extract taken out to part I.

A_p = amounts (nmol) of added ^{14}C-labelled steroid precursor.

The calculation of total radioactivity from the precursor incorporated into the ^{14}C-labelled estrone is determined according to the formula:

$$\frac{R_1 \times D_i}{n} \quad \text{d.p.m.}$$

Percentage of the added ^{14}C-labelled precursor converted to estrone:

$$= \frac{R_1 \times D_i}{n} \times \frac{1}{D_p} \times 100$$

Amounts of ^{14}C-labelled estrone formed:

$$= \frac{A_p \times R_1 \times D_i}{n} \times \frac{1}{D_p} \quad \text{nmol}$$

Part II. <u>Determination of specific activities, total productions (nmol) and endogenous productions (nmol) of steroid metabolites</u>

Exact aliquot of the total extract does not necessarily have to be known here. The aqueous fraction was extracted according to the method described in extraction and was acetylated with ^{3}H-labelled acetic anhydride of known specific activity. After the extraction of the acetylation mixture 20 μg each of the appropriate non-radioactive steroid acetates supposed to have been formed in the incubation, were added to the methylene chloride extract which was quantitatively applied to TLC 6. The acetylated ^{14}C--labelled steroids were, as in part I, separated and purified until a ^{14}C : ^{3}H constant ratio was attained, through successive steps of chromato--graphy. From the ^{14}C : ^{3}H ratios, the specific activity of the steroid under

study at the end of incubation is calculated as follows:

Estimation of specific activities of isolated steroids:

Taking estrone as an example, let

S_a = specific activity of ^3H-acetic anhydride (d.p.m./nmol).

R_2 = constant $^{14}C/^3H$ ratio of isolated doubly labelled estrone mono-acetate.

Then, specific activity of estrone = $\frac{R_2 \times S_a}{2}$ d.p.m./nmol.

Determination of total and endogenous yields of an isolated steroid product:

The total amount in nano moles (endogenous plus conversion from the added precursor) of an isolated steroid was obtained simply by dividing the total radioactivity in d.p.m., associated with that compound (part I) by its respective specific activity (part II) in d.p.m./nmol.

The endogenous production (nmol) was estimated by subtracting the quantity (nmol) coming from the conversion of the precursor (part I) of the total amount(nmol).

V . RESULTS

Each incubation involving elaborate procedures for the characterization and quantification of several steroids, it was not possible to duplicate the results within the same experiment. However, the same results were duplicated in separate experiments. The results consistantly agreed as to their relative values.

Section 1

Studies with human term placenta

The characterization of individual steroids is reported in Section 1 Chapter IV, of this thesis.

Studies with term placenta were carried out either on minces, homogenates or subcellular fractions and are reported in three different chapters.

Chapter I

Placental minces

A) Effect of HCG on the aromatization of 4-^{14}C-androstenedione and 4-^{14}C-testosterone by placental minces:

A preliminary study was undertaken in order to investigate the possible action of HCG on the aromatization of C-19 steroids by term placenta. Cedard et al (109) demonstrated in 'in vitro' perfusion of term placenta that HCG significantly improves the aromatization of neutral steroids, such as androstenedione, dehydroisoandrosterone and testosterone. However, attempts to stimulate the secretion of estrogens 'in vivo' by the administration of HCG either after the third month of pregnancy (276) or at term (277) were

unsuccessful.

The apparent contradiction of these observations prompted us to investigate further the action of HCG on the aromatization of steroids.

Experimental conditions

Minces of human term placenta (one gram tissue per incubation flask) were preincubated in 5 ml KRBG for one hour at 37°C, as described under methods (page 44-45). At the end of preincubation period, the preincubation medium was discarded and the minces transferred to another set of flasks containing 4-^{14}C--androstenedione (1,814,800 d.p.m., 4.0 μg) or 4-^{14}C-testosterone (1,922,400 d.p.m., 4.2 μg) in a final volume of 6 ml KRBG. HCG was added at the concentra--tion of either 500 I.U., or 3000 I.U. to each of the experimental flasks. The incubations were then allowed to proceed for two hours.

Results and discussion

As indicated in methods (page 49) after the incubation, the media plus the washings of the tissue and the minced tissue were processed separately. In Table III however, the percentage conversion of 4-^{14}C-androstenedione as well as of 4-^{14}C-testosterone to various steroids are expressed in terms of the media plus tissue.

The pattern of conversion products obtained when 4-^{14}C-androstene--dione or 4-^{14}C-testosterone was used in separate incubations was very similar. When 4-^{14}C-androstenedione was used as a precursor, the testosterone, estrone and estradiol were obtained as conversion products. The use of 4-^{14}C-testosterone resulted in the formation of ^{14}C-labelled androstenedione, estrone and estra--diol. Addition of HCG (500 or 3000 I.U.) resulted in a decreased percentage conversion of each precursor to the products. Estriol could not be isolated in the studies using either precursor. This observation is similar with the

TABLE III

EFFECT OF HCG ON THE AROMATIZATION OF 4-^{14}C-ANDROSTENEDIONE AND 4-^{14}C-TESTOSTERONE BY PLACENTAL MINCES

Precursor added		% Conversion of ^{14}C-precursor to: TEST●	ASD	E_1	E_2
^{14}C-ASD	Control	3.5	*51.5	15.2	6.8
	HCG (500 I.U)	3.0	*56.1	12.2	5.1
	HCG(3000 I.U)	2.0	*61.7	7.5	3.1
^{14}C-TESTO	Control	*2.5	45.3	27.7	11.0
	HCG (500 I.U)	*2.0	62.6	14.4	8.7
	HCG(3000 I.U)	*2.0	60.8	11.4	7.3

* Unconverted substrate recovered.

findings of Jackanicz and Diczfalusy (93) and of Bagett et al (92). From the data, it is quite apparent that the placental preparations convert more testo--sterone to androstenedione than vice versa (74-76). Furthermore, the results indicate that the aromatization of testosterone is quantitatively greater than that of androstenedione. As shown in Table III, the percentage conversion of testosterone to estrone (27.7%) and estradiol (11%) was twice as high as that of androstenedione under similar experimental conditions (estrone: 15.2%, estradiol: 6.8%).

In the presence of 500 I.U. of HCG, there was a slight decrease in the aromatization of androstenedione and testosterone to estrone and estradiol. However, a significant decrease was observed when the concentration of HCG was increased to 3000 I.U. In this instance, when androstenedione was the precursor, the formation of estrone (7.5%) and estradiol (3,1%) were decreased to 50% of control values. An almost similar decrease in the formation of estrone and estradiol was observed when testosterone was the substrate.

It should be pointed out that the aromatizing ability of placental minces used in this experiment was higher than in subsequent experiments.

B) Effect of HCG on the metabolism of 4-^{14}C-testosterone by placental minces in the presence of NADP plus G-6-P:

Our preliminary studies indicated an inhibitory effect of HCG on placental aromatizing systems. Previously Cedard et al (111) have suggested that HCG may act by increasing the availability of NADPH, which in turn accele--rates the hydroxylation processes and aromatization of ring A. In view of this, it became of interest to see if the aromatizing activity in placental minces is increased in the presence of an NADPH generating system and whether addition of HCG would either enhance or decrease this effect.

Experimental conditions

Three incubation flasks, each containing an aliquot of one gram of placental minces were preincubated for one hour. The tissues were then trans--ferred to another set of flasks, containing 4-^{14}C-testosterone (1,920,200 d.p.m., 4.2 μg) in a total volume of 6 ml of KRBG. Incubations were carried out for two hours. The incubation flasks were supplemented as follows:

flask 1. control, no additives.

flask 2. NADP (2.6 μmol), G-6-P (8.2 μmol).

flask 3. NADP (2.6 μmol), G-6-P (8.2 μmol), HCG (3000 I.U.).

Results and discussion

As indicated previously (page 49); after the incubation, the media plus tissue washings and the tissue minces were processed separately. Table IV presents the percentage conversions of 4-^{14}C-testosterone to different steroids in the media, in the tissue as well as in the sum of media plus tissue.

Besides androstenedione, estrone, and estradiol, 19-hydroxy-androst--enedione was also isolated and charecterized. This latter compound was consis--tantly detected in all subsequent studies. Since tritiated 19-hydroxy-androst--enedione is not commercially available, its exact losses during the course of extraction and purification procedures could not be established. However, the values were corrected using the mean losses of other metabolites, i.e., androstenedione, estrone and estradiol.

The percentage conversion of testosterone to individual metabolites obtained in the presence of a NADPH generating system was very different from that observed in the absence of co-factors. Considering the percentage conver--sion of testosterone by the media plus tissue (Table IV), addition of NADP plus G-6-P to the incubation of placental minces resulted in a decreased

TABLE IV

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY PLACENTAL MINCES IN THE PRESENCE OF NADP+G-6-P

% Conversion from ^{14}C-testosterone to:		Media	Tissue	Media+Tissue
*TESTO	Control	0.9	0.5	1.4
	NADP + G-6-P	0.5	0.6	1.1
	NADP + G-6-P + HCG	0.3	0.4	0.7
ASD	Control	44.0	21.0	65.0
	NADP + G-6-P	1.6	1.0	2.6
	NADP + G-6-P + HCG	1.7	0.7	2.4
E_1	Control	1.3	3.7	5.0
	NADP + G-6-P	18.3	37.6	55.9
	NADP + G-6-P + HCG	8.4	35.3	43.7
E_2	Control	0.9	0.8	1.7
	NADP + G-6-P	8.8	2.1	10.9
	NADP + G-6-P + HCG	23.3	7.2	30.5
19-OH-ASD	Control	0.9	0.5	1.4
	NADP + G-6-P	0.7	0.3	1.0
	NADP + G-6-P + HCG	0.5	0.2	0.7

* Unconverted substrate recovered.

conversion of testosterone to androstenedione (2.6%) and 19-hydroxy-androst--enedione (1.0%) when compared to control values (androstenedione: 65.0%, 19-hydroxy-androstenedione: 1.4%). However, the conversion of the substrate to estrone (56%) and estradiol (11%) was much greater with the NADPH generating system than without co-factors (estrone: 5%, estradiol: 1.7%).

Addition of HCG to the incubation flasks simultaneously with an NADPH generating system, resulted in a three fold increase in the conversion of testosterone to estradiol (30.5%) which was partially compensated by a lower conversion to estrone (43.7%). From the analysis of the different metabolites in the media and in the tissue, it is interesting to note that in control as well as in experimental studies, the quantity of androstenedione, estradiol and 19-hydroxy-androstenedione recovered in the media exceeded that obtained in the corresponding tissues. In contrast, the yield of estrone was always higher in tissues than in the corresponding media.

These observations confirm the findings of our previous study; i.e., the ability of placental minces to convert 4-^{14}C-testosterone to androstene--dione, estrone and estradiol. The isolation of 19-hydroxy-androstenedione suggests that it might be an intermediary in the formation of estrogens. It has been reported that in the placenta, the conversion of androstenedione to estrone and estradiol takes place via 19-hydroxy-androstenedione (83).

Addition of an NADPH generating system to the placental minces, increased estrogen production from testosterone. Cedard et al (111) reported similar observations in 'in vitro' perfusion of term placenta. It must be emphasized that in the presence of an NADPH generating system, HCG does not inhibit aromatization of the precursor, but leads to a shift in the formation of estrone to estradiol.

C) - (i) and (ii); Effect of HCG on the metabolism of 4-^{14}C-testosterone by placental minces in the presence of various co-factors:

Experiments with placental minces were undertaken in order to know if either G-6-P or NADP or both were required to cause the shift reported in the formation of estrone to estradiol, when HCG was present. At the same time cyclic 3′,5′-AMP was tested in the presence of HCG. It has been suggested that tropic hormones (such as ACTH and LH) act by increasing initially the intra-cellular concentration of cyclic 3′,5′-AMP, which in turn increases the cellular NADPH concentration (111). Since it is believed (278) that 3′,5′-AMP does not penetrate the cell membrane easily, the substituted derivative of this nucleotide (i.e., 3′,5′-AMP dibutyryl) was also used, which seems to penetrate the cell membrane easily (279).

Experimental conditions

One gram of preincubated placental minces (see methods) were incubated for two hours at 37°C with 4-^{14}C-testosterone (1,793,600 d.p.m., 4.0 μg) in a total volume of 6 ml KRBG. Two studies were conducted; C- (i) and C- (ii). Each study consisted of seven incubation flasks supplemented as follows:

C- (i). flask 1. control.
flask 2. G-6-P (8.2 μmol).
flask 3. G-6-P (8.2 μmol), HCG (3000 I.U.).
flask 4. 3′,5′-AMP (30.4 μmol).
flask 5. 3′,5′-AMP (30.4 μmol), HCG (3000 I.U.).
flask 6. 3′,5′-AMP D.B. (30.4 μmol).
flask 7. 3′,5′-AMP D.B. (30.4 μmol), HCG (3000 I.U.).

C- (ii). flask 1. control.

flask 2. NADP (2.6 μmol).

flask 3. NADP (2.6 μmol), HCG (3000 I.U.).

flask 4. NADP (2.6 μmol), 3′,5′-AMP (30.4 μmol).

flask 5. NADP (2,6 μmol), 3′,5′-AMP (30.4 μmol), HCG (3000 I.U.).

flask 6. NADP(2.6 μmol), G-6-P (8.2 μmol).

flask 7. NADP (2.6 μmol), G-6-P (8.2 μmol), HCG (3000 I.U.).

Results and discussion

In Table V, it can be seen that the addition of either G-6-P or 3′,5′-AMP or its dibutyryl derivative did not affect the conversion of 4-^{14}C--testosterone to androstenedione, estradiol and 19-hydroxy-androstenedione above the values obtained for the control. However, there was a slight decrease in the conversion to estrone, which was further enhanced by the simultaneous addition of HCG.

One can see in Table VI, that when placental minces were supplemented with NADP, the yield of androstenedione was decreased from 79.1% to 46.4%. This was accompanied by an increase of testosterone conversion to estrone (32%), estradiol (3.4%) and 19-hydroxy-androstenedione (8.9%). The respective values obtained in control studies were estrone: 6.4%, estradiol: 0.8%, and 19-hydroxy--androstenedione: 1.8%.

The addition of 3′,5′-AMP with NADP did not alter the conversion of testosterone to different metabolites significantly, when compared to control values.The addition of HCG with NADP or with NADP plus 3′,5′-AMP, led to a decrease of testosterone conversion to androstenedione (28.4% and 14.8% res--pectively) and 19-hydroxy-androstenedione (6.7% and 5.9% respectively), accompanied by an increased conversion to estrone (50.2% and 56.0% respectively) and estradiol (8.6% and 16.9% respectively).

When G-6-P was added simultaneously with NADP to the incubation of

TABLE V

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESOSTERONE BY PLACENTAL MINCES IN THE PRESENCE OF VARIOUS CO-FACTORS

	% Conversion from ^{14}C-testosterone to:				
	*TESTO	ASD	E_1	E_2	19-OH-ASD
Control	1.4	72.7	5.4	1.2	1.5
G-6-P	1.4	67.6	4.4	1.3	1.5
G-6-P + HCG	1.6	67.8	3.6	1.4	1.3
3',5'-AMP	1.3	71.6	4.2	1.3	1.2
3,5-AMP + HCG	1.9	70.6	1.8	1.6	0.8
3',5'-AMP D.B	1.6	65.3	3.5	0.8	1.1
3',5'-AMP D.B + HCG	1.5	69.3	2.4	0.9	0.9

* Unconverted substrate recovered.

TABLE VI

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY PLACENTAL MINCES IN THE PRESENCE OF VARIOUS CO-FACTORS

		%-Conversion from ^{14}C-testosterone to:			
	*TESTO	ASD	E_1	E_2	19-OH-ASD
Control	0.8	79.1	6.4	0.8	1.8
NADP	1.3	46.4	32.0	3.4	8.9
NADP+ HCG	1.5	28.4	50.2	8.6	6.7
NADP + 3′,5′-AMP	1.3	42,9	35.0	5.7	10.9
NADP + 3′,5′-AMP + HCG	1.3	14.8	56.0	16.9	5.9
NADP + G-6-P	0.6	1.5	86.2	8.8	0.7
NADP + G-6-P + HCG	0.9	1.1	75.1	21.6	0.8

* Unconverted substrate recovered.

placental minces, a decrease in the formation of androstenedione (to 1.5%) was accompanied by an increase of estrone, estradiol. Addition of HCG with NADP plus G-6-P, caused a shift in the formation of estrone (75.1%) to estradiol (21.6%); similar to that already observed in chapter I (B).

Since no other estrogens than estrone and estradiol could be detected, the sum of the conversions from testosterone was calculated to obtain the total aromatization products. To see if the effect of HCG would not be on the 17β-hydroxyl group of the steroids, the sum of the percentage of the different 17β-hydroxy-steroids (testosterone plus estradiol) present in the incubations was also calculated. One can see in Table VII that addition of either G-6-P or 3',5'-AMP or its dibutyryl derivative alone or each in combination with HCG, does not affect the relative concentrations of steroids possessing a 17β-hydroxyl group or the extent of aromatization.

Figure 4 is a graphic representation of the action of HCG on 17β-hydroxy-steroids and aromatization products in study C-(ii). The total conversion of 17β-hydroxy-steroids increased from a value of 1.6 to 5.0%, when NADP was present. There was a further increase by simultaneous addition of 3',5'-AMP (7.0%) or G-6-P (9.4%). In each case, these conversions were significantly increased by HCG (10.0%, 18.2% and 22.5% respectively).

NADP or NADP in the presence of either 3',5'-AMP or G-6-P, increased the total yield of aromatized products (35.0%, 40.7% and 95.0% respectively) over control values (7.2%). The presence of HCG, further enhanced these conversions (59.0%, 72.9% and 96.7% respectively).

Since in the presence of a NADPH generating system the aromatization of testosterone was maximally stimulated (95%), the effect of HCG on aromati--zation was difficult to assess (97%).

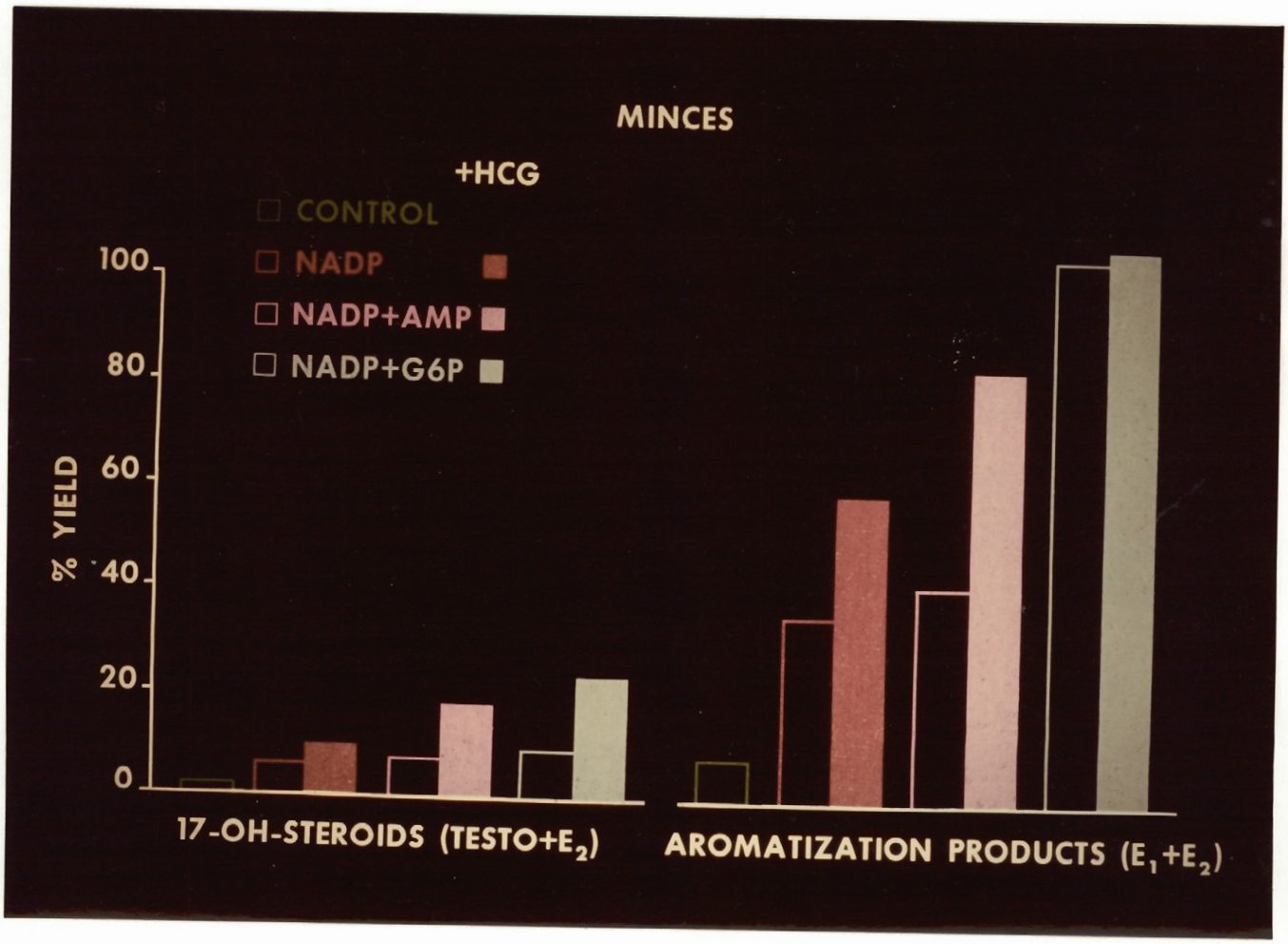
TABLE VII

EFFECT OF HCG ON TOTAL 17β-OH-STEROIDS AND AROMATIZATION PRODUCTS FROM 4-^{14}C-TESTOSTERONE BY PLACENTAL MINCES IN THE PRESENCE OF VARIOUS CO-FACTORS

	% Conversion of ^{14}C-precursor to:	
	17β-OH-Steroids (TESTO + E_2)	Aromatization products (E_1 + E_2)
Control	2.6	6.6
G-6-P	2.7	5.7
G-6-P + HCG	3.0	5.0
3',5'-AMP	2.6	5.5
3',5'-AMP + HCG	3.5	3.4
3',5'-AMP D.B	2.4	4.3
3',5'-AMP D.B + HCG	2.4	3.3

FIGURE 4

GRAPHIC REPRESENTATION OF THE ACTION OF HCG ON 17β-HYDROXY-STEROIDS (TESTO--STERONE PLUS ESTRADIOL) AND AROMATIZATION PRODUCTS (ESTRONE PLUS ESTRADIOL); PLACENTAL MINCES INCUBATED IN THE PRESENCE OF VARIOUS CO-FACTORS.



C) - (iii); Effect of HCG in the presence of various co-factors on the production of steroids from 4-^{14}C-testosterone by placental minces:

This study was a part of study C-(ii). The production of steroids, particularly of estrone and estradiol was measured in the incubation mixtures according to the procedure described in methods (page 53-56 and Figure 3).

The percentage conversion of testosterone to different steroids (Table VI), the specific activity of individual steroids and their endogenous productions were calculated.

When the radioactivity present in certain ^{14}C-steroids was very small as determined from their percentage conversion, known amounts of d.p.m./nmol of pure ^{14}C-steroids were added to the corresponding extracts of part II before acetylation with ^{3}H-acetic anhydride (S.A. : 135,000 d.p.m/nmol) In this way ^{14}C : ^{3}H ratios could be established. From these ratios, the quantity (nmol) of steroids present could be established, after the substraction of the amount of ^{14}C-steroid tracer added.

To determine the amount of steroids present at the begining of the incubations, placental minces were preincubated for one hour and were processed without further incubation. The steroids were measured by a modification of the double isotope derivative assay of Stachenko et al (271).

Results and discussion

The results are presented in Table VIII and IX.

Considering the control I values, which gives an idea of the quantity of steroid present at the begining of the incubation, one can see that estrone was present in highest amounts (1.2 nmol) followed by testosterone (0.5 nmol), and estradiol (0.1 nmol).

When the quantities of steroids in control I were compared to control II (this giving the extent of steroid synthesis from endogenous substrates),

TABLE VIII

EFFECT OF HCG ON THE PRODUCTION OF ESTRONE, ESTRADIOL, AND ESTRONE+ESTRADIOL (in nmol) FROM 4-^{14}C-TESTOSTERONE BY PLACENTAL MINCES IN THE PRESENCE OF VARIOUS CO-FACTORS

	E_1 (n mol)			E_2 (n mol)			E_1+E_2 (n mol)		
	T.P. —	Ex. P. =	En. P.	T.P. —	Ex. P. =	En. P.	T.P —	Ex. P. =	En. P.
Control I	-	-	1.2	-	-	0.1	-	-	1.3
Control II	2.4	0.9	1.5	0.2	0.1	0.1	2.6	1.0	1.6
NADP	5.7	4.4	1.3	0.5	0.4	0.1	6.2	4.8	1.4
NADP+ HCG	9.0	6.9	2.1	1.1	1.0	0.1	10.1	7.9	2.2
NADP+ 3′,5′-AMP	5.5	4.8	0.7	0.9	0.6	0.3	6.4	5.4	1.0
NADP+ 3′,5′-AMP+ HCG	9.2	7.7	1.5	2.3	2.1	0.2	11.5	9.8	1.7
NADP+ G-6-P	12.6	11.8	0.8	1.4	1.1	0.3	14.0	12.9	1.1
NADP+ G-6-P+ HCG	11.6	10.6	1.0	2.6	2.2	0.4	14.2	12.8	1.4

I . This control represents the amount of steroids present at the begining of the incubation, when minces were preincubated for one hour without any further incubation.

II . This control is the experimental control, where incubations were carried out with the substrate but without any co-factors.

T.P. — Total production, Ex. P. — Exogenous production, En. P. — Endogenous production.

TABLE IX

EFFECT OF HCG ON THE PRODUCTION OF TESTOSTERONE (in nmol) BY PLACENTAL MINCES IN THE PRESENCE OF VARIOUS CO-FACTORS

	TESTO (nmol)				
	T.P.	—	Ex.P.	=	En.P.
Control I	-		-		0.5
Control II	0.6		0.1		0.5
NADP	0.6		0.2		0.4
NADP + HCG	0.7		0.2		0.5
NADP + 3′,5′-AMP	0.6		0.2		0.4
NADP + 3′,5′-AMP + HCG	0.7		0.2		0.5
NADP + G-6-P	0.6		0.1		0.5
NADP + G-6-P + HCG	0.6		0.1		0.5

I . This control represents the amount of steroids present at the beginning of the incubation, when minces were preincubated for one hour without any further incubation.

II. This control is the experimental control, where incubations were carried out with the substrate, but without any co-factors.

T.P. — Total production, Ex. P. — Exogenous production, En. P. — Endogenous production.

it can be concluded that very little, if any steroids were produced during the the two hour incubation of the placental minces. Addition of any of the co-factors alone or in combination with HCG, showed no significant change in the endogenous productions of estrone, estradiol or testosterone.

These results demonstrate that there is no 'de novo' production of steroids during incubation of the placental minces. These findings are in agreement with the observations of Cedard et al (281) in perfusion studies of term placenta.

D) Effect of HCG on the metabolism of 4-^{14}C-testosterone by placental minces incubated at different time intervals:

Since a lag period has been reported in the action of HCG (112,282), placental minces were incubated for various lengths of time starting at 5 min--utes up to 4 hours, to investigate this delayed action of HCG.

Experimental conditions

One gram of placental minces was incubated with 4-^{14}C-testosterone (1,782,900 d.p.m., 3.9 μg) in a total volume of 6 ml KRBG for each incubation flask. Two experiments were carried out. Experiment 1 consisted of 5 sets of 3 incubation flasks. Each set was incubated for 5 different lengths of time. Each set of incubation flasks was as follows:

flask 1. control, no additives.

flask 2. NADP (2.6 μmol).

flask 3. NADP (2.6 μmol), HCG (3000 I.U.).

Incubations were carried out for 5,20,40,120, and 240 minutes respectively at 37^{o}C.

Experiment 2 consisted of 2 sets of 3 incubation flasks. Each set was incubated for 20 or 120 minutes and was set up as follows:

flask 1. control, no additives.

flask 2. NADP (2.6 μmol), G-6-P (8.2 μmol).

flask 3. NADP (2.6 μmol), G-6-P (8.2 μmol), HCG (3000 I.U.).

Results and discussion

The results of experiment 1 are indicated in Table X, which shows the percentage conversion of 4-^{14}C-testosterone to different metabolites in the media plus tissues.

One can see in Table X, as well as in Figure 5 that most of the ^{14}C-testosterone is already utilized after 5 minutes, only 11.4% being left unconverted in control studies and 6.8% and 6.4% with NADP or NADP plus HCG.

Most of the ^{14}C-testosterone in the control was converted to androstenedione (80%) after 5 minutes (Figure 5). This quantity did not fluctuate up to 4 hours (82%). When NADP was present in the incubation mixture the conversion to androstenedione was very rapid. At 5 minutes 71% of the radioactivity of testosterone was incorporated in androstenedione, but with time androstenedione was utilized further and only 36% was left at the end of 4 hours incubation. Up to 40 minutes the pattern of conversion to androstene--dione was very similar when HCG was added simultaneously with NADP, thereafter the utilization of androstenedione was much enhanced. Only 18% was left after 4 hours.

In the control (Figure 6) after 5 minutes, the conversion of the precursor to estrone (5.2%) and estradiol (1.1%) did not change significantly and it remained same up to 4 hours (estrone: 7.6%, estradiol: 1.2%). After 5 minutes in the presence of NADP with or without further addition of HCG, there was a significant increase in the formation of estrone over the control, the conversion of estradiol being only slightly increased. As the time of incubation was prolonged, there was an increased conversion of testosterone to

TABLE X

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY PLACENTAL MINCES INCUBATED AT DIFFERENT TIME INTERVALS

% Conversion from ^{14}C-testo--sterone to:		Time incubated 5 min.	20 min.	40 min.	120 min.	240 min.
*TESTO	Control	11.4	2.4	1.2	0.8	1.6
	NADP	6.8	2.0	2.4	1.4	3.4
	NADP + HCG	6.4	2.5	2.4	1.5	4.2
ASD	Control	80.1	85.5	87.0	79.1	81.5
	NADP	70.7	68.2	51.8	46.9	36.3
	NADP + HCG	77.0	71.4	59.1	28.4	17.6
E_1	Control	5.2	5.2	5.1	6.4	7.6
	NADP	15.1	18.0	24.9	32.0	34.3
	NADP + HCG	9.5	16.4	20.7	50.2	54.1
E_2	Control	1.1	0.9	0.6	0.8	1.2
	NADP	2.0	2.1	2.8	3.1	3.7
	NADP + HCG	1.4	2.4	2.9	8.6	8.8
19-OH-ASD	Control	1.3	1.5	1.3	1.8	1.8
	NADP	5.5	8.1	8.2	8.6	9.8
	NADP + HCG	4.2	7.4	8.0	10.1	8.3

* Unconverted substrate recovered.

FIGURE 5

EFFECT OF HCG ON THE PERCENTAGE CONVERSION OF ANDROSTENEDIONE AND TESTOSTERONE; PLACENTAL MINCES INCUBATED IN THE PRESENCE OF NADP FOR DIFFERENT LENGTHS OF TIME.

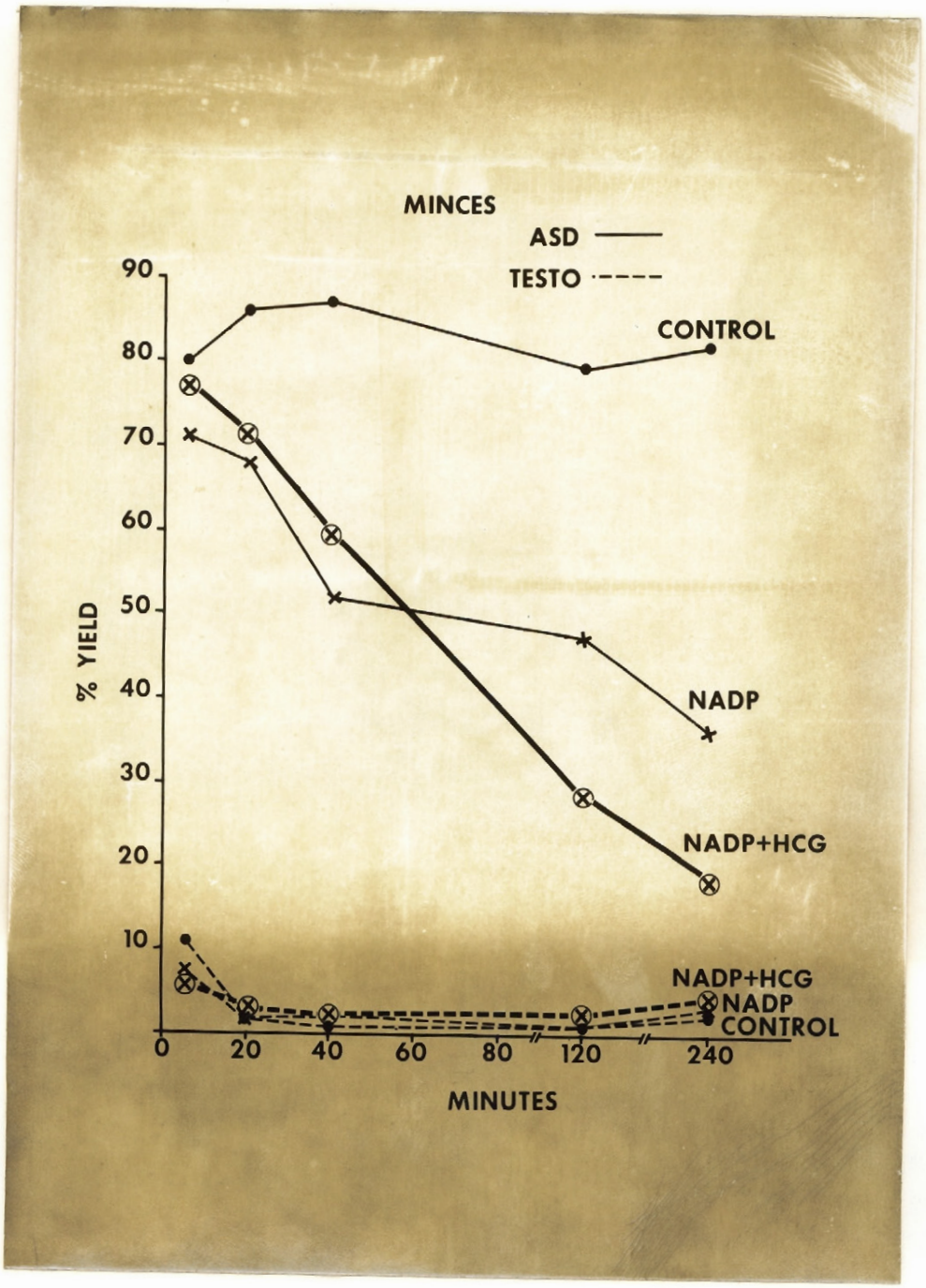
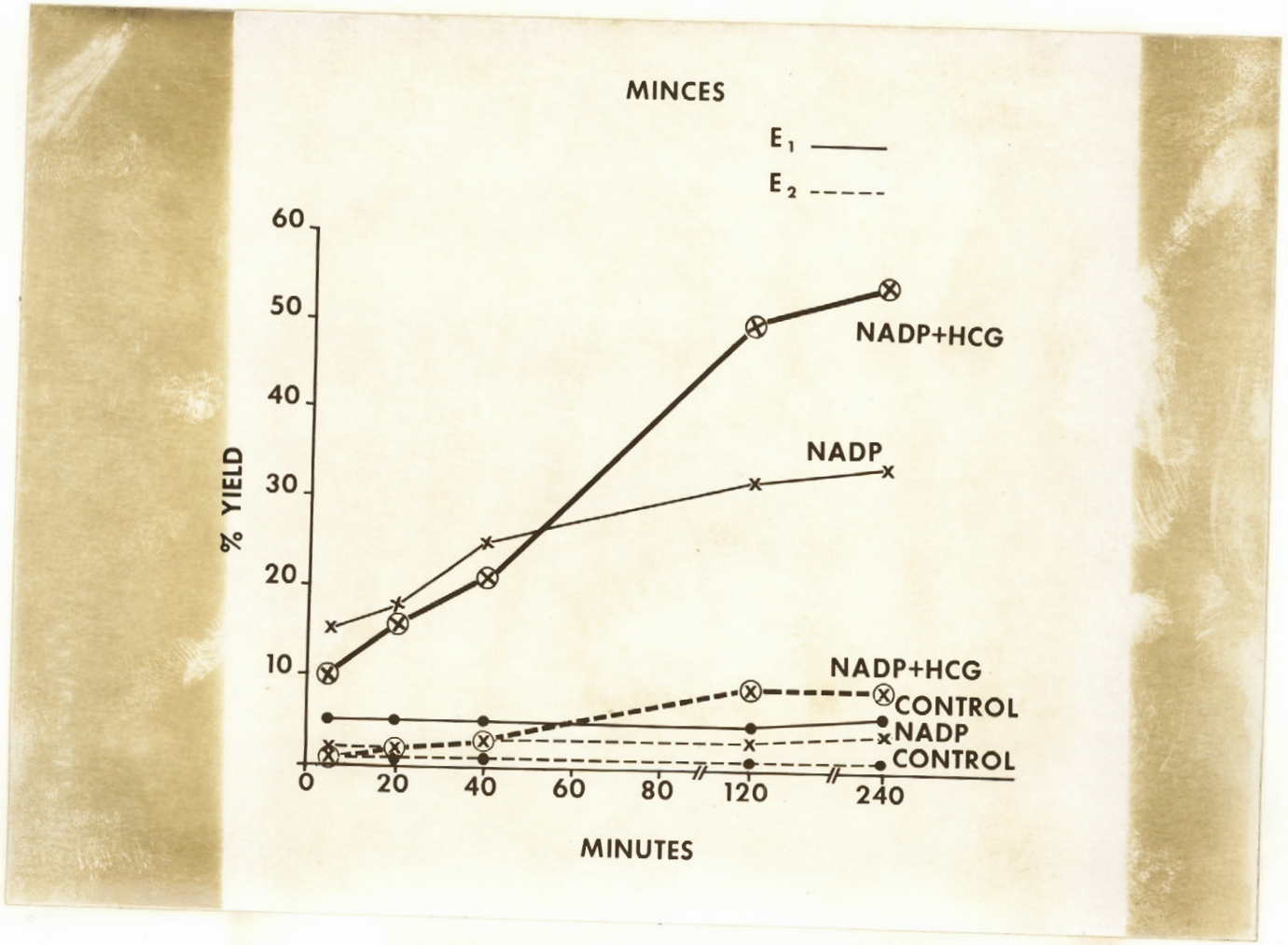


FIGURE 6

EFFECT OF HCG ON THE PERCENTAGE CONVERSION OF ESTRONE AND ESTRADIOL; PLACENTAL MINCES INCUBATED IN THE PRESENCE OF NADP FOR DIFFERENT LENGTHS OF TIME.



estrone and estradiol. It can be noticed however, that up to 40 minutes of incubation, the values of the conversion to estrone and estradiol with NADP plus HCG were smaller than those observed with NADP alone. However, at 2 and 4 hours when HCG was present in the incubation with NADP, the conversion values were significantly higher than with NADP alone.

Figure 7 presents graphically the percentage conversion of testo--sterone to estrone plus estradiol in placental minces as a function of incubation time. Here, the delayed action of HCG is clear. When NADP is present, the addition of HCG decreased the conversion to estrone plus estradiol up to 40 minutes, but after 2 hours the reverse took place.

As indicated in methods (page 49), after the incubation, the media and the minced tissues were processed separately. Table XI presents the conversion ratios of testosterone to different metabolites calculated as in the media/in the tissues.

The ratios obtained for testosterone, androstenedione, estradiol and 19-hydroxy-androstenedione were always higher than 1, regardless of the co-factor present or the time of incubation. In the instance of estrone however, these ratios were consistantly lower than 1 (0.2 to 0.7). These data confirm our earlier observations presented in this thesis (chapter I (B), Table IV).

In experiment 2, incubations were carried out for 20 minutes and for 2 hours in the presence of NADP plus G-6-P or NADP plus G-6-P plus HCG. The data obtained after 2 hours of incubation have been reported in chapter I (B), Table IV. The conversion to estrone and estradiol obtained at 20 minutes of incubation showed that HCG promoted a two fold increase in the conversion to estradiol, which was compensated by a simultaneous decrease in estrone.

The sums of the percentage conversion to estrone plus estradiol are

FIGURE 7

GRAPHIC REPRESENTATION OF THE ACTION OF HCG ON AROMATIZATION OF TESTOSTERONE TO ESTRONE PLUS ESTRADIOL; PLACENTAL MINCES INCUBATED IN THE PRESENCE OF NADP FOR DIFFERENT LENGTHS OF TIME.

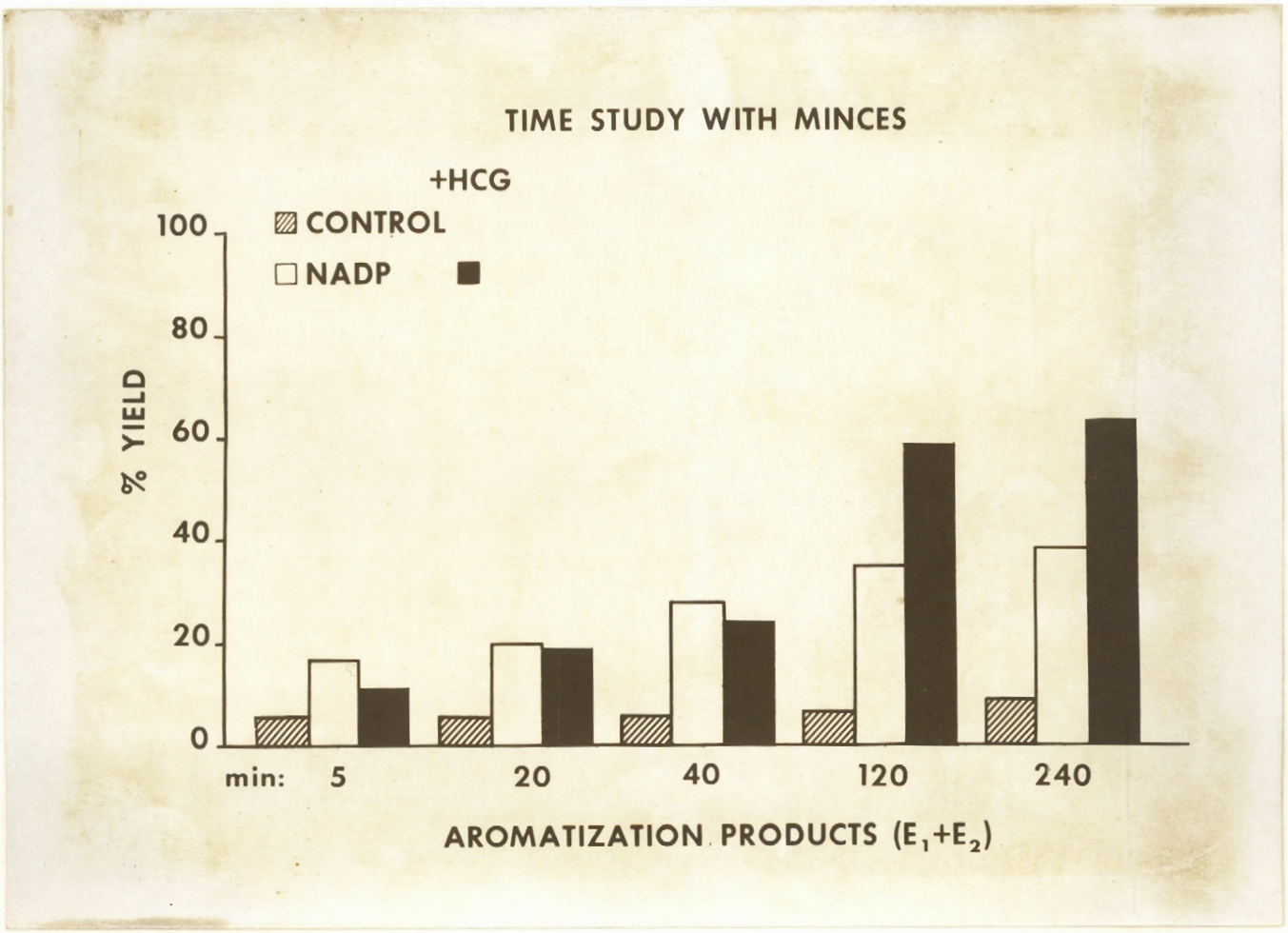


TABLE XI

RATIOS OF MEDIA OVER TISSUE OBTAINED IN DIFFERENT METABOLITES OF 4-^{14}C-TESTOSTERONE INCUBATED WITH PLACENTAL MINCES FOR DIFFERENT LENGTHS OF TIME

		Ratios : media/tissue		
		20 min	40 min	120 min
TESTO	Control	3.8	1.4	1.0
	NADP	5.7	1.2	1.3
	NADP + HCG	5.3	1.4	1.5
ASD	Control	2.6	2.8	2.7
	NADP	2.9	3.1	2.9
	NADP + HCG	4.0	4.4	3.3
E_1	Control	0.7	0.6	0.4
	NADP	0.3	0.4	0.5
	NADP + HCG	0.2	0.3	0.3
E_2	Control	2.0	2.0	1.7
	NADP	2.5	2.5	3.4
	NADP + HCG	1.7	1.4	3.3
19-OH-ASD	Control	0.7	0.9	1.3
	NADP	3.5	3.6	3.8
	NADP + HCG	2.9	4.0	4.5

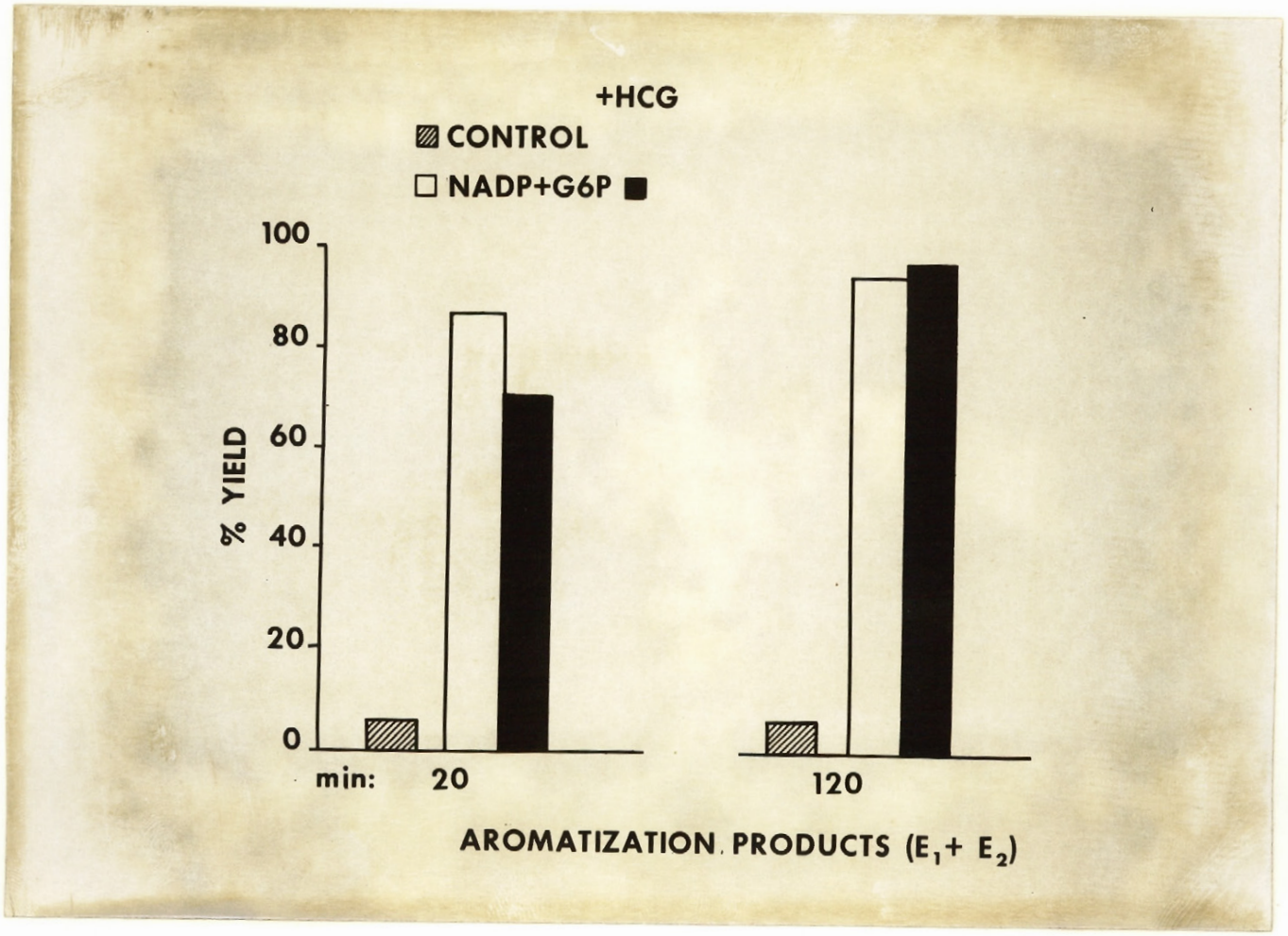
shown in Figure 8. Once more, it can be seen that at 20 minutes, the conversion to estrone plus estradiol was higher with NADP plus G-6-P (87%) than with NADP plus G-6-P plus HCG (71%). However, at 2 hours, the conversion with NADP plus G-6-P plus HCG reached values (97%) comparable to those obtained with NADP plus G-6-P (95%).

From these time studies, it can be concluded that at the beginning of the incubation, in the presence of NADP or of an NADPH generating system, the action of HCG on the conversion of testosterone to estrone plus estradiol is slightly inhibitory; but after 2 hours, the overall effect is stimulatory. These observations seem to agree with the findings of Toren et al (112), who studied the metabolic actions of placental hormones (such as HCG and estradiol) on citric acid cycle enzymes. In perfusion studies of the placenta, they showed a definite lag period of 5 hours before observing the stimulatory action of HCG plus estradiol on the utilization of citrate. A longer lag period was noted when HCG alone was used. Pincus (282) had reported a several hour lag period for the effects of gonadotrophin on steroidogenesis by the perfused placenta. Our results however are partly in contradiction with the findings of Cedard et al (111,281), who found a maximal conversion of testosterone to estrone and estradiol during the first half hour of perfusion. Simultaneous addition of HCG and testosterone at the beginning of the experiment or 1 hour after, stimulated the formation of aromatized products very significantly (111, 281). In our control studies, the conversion of testosterone to estrone and estradiol was small and almost maximal at 5 minutes. Our studies with HCG showed a lag period of 1 hour before the increased formation of estrogens was evident (Figure 6).

Analysis of individual steroids in the media and the tissues showed that all the steroids isolated were in higher concentration in the media than

FIGURE 8

GRAPHIC REPRESENTATION OF THE ACTION OF HCG ON AROMATIZATION OF TESTOSTERONE TO ESTRONE PLUS ESTRADIOL; PLACENTAL MINCES INCUBATED IN THE PRESENCE OF NADP PLUS G-6-P FOR 20 OR 120 MINUTES.



in the tissues; with the exception of estrone.Saure (283) was also able to isolate more androstenedione from the medium than from the tissue following incubation of slices of human term placentas with testosterone. 'In situ' perfusion of midterm placentas with androstenedione, testosterone, 19-nor--testosterone, dehydroisoandrosterone and dehydroisoandrosterone sulfate have resulted in the formation of estrone and estradiol, but irrespective of the precursor administered three times more estrone than estradiol was isolated from the perfusates and three times more estradiol than estrone from the placental tissue (81,94, 70, 71). Studies conducted by Stern (284) on placental aromatization of 15α-hydroxy steroids also showed the presence of larger amounts of 15α-hydroxy-estradiol in the placenta than in the perfusate. The author concluded that the steroid is bound to placental tissue. Such binding might be associated with a physiological role of this steroid in the placenta (284).

Discussion

The different studies reported here with placental minces show that androstenedione and testosterone can be converted to estrogens. This conversion is limited when placental minces are not supplemented with co-factors. When the incubation media are supplemented with HCG, G-6-P, or 3′,5′-AMP alone, or G-6-P or 3′,5′-AMP in combination with HCG, there is a decreased conversion of testosterone to estrone and estradiol. Addition of NADP alone to the incu--bation media results in a significant increase in the formation of estrone and estradiol. This would suggest that in such tissues the supply of NADP is not sufficient or is not readily available, whereas G-6-P is relatively more readily available. Under our experimental conditions, when placental minces were supplemented with G-6-P, besides NADP, the aromatization of ^{14}C-testosterone

was almost complete (95%).

The increase in the aromatizing activity induced by NADP was enhanced by the addition of HCG, but not to the same extent as with G-6-P plus NADP. The data suggest that one of the actions of HCG could be to increase the pro--vision of G-6-P, which in turn in the presence of NADP, generates more NADPH for steroid hydroxylation and aromatization.

Furthermore, it seems that this generation of G-6-P by HCG, could be limited or regulated in the cells; as aromatization of the testosterone molecule was only 59% with NADP plus HCG, but reached 95% when G-6-P was added directly with NADP to the placental minces. Cellular metabolism is regulated by a chain of reactions;it can be conceived that when fed directly to the enzyme systems, G-6-P has a much faster effect on placental aromatizing systems than when it is produced endogenously.

The delayed action of HCG on the aromatization of testosterone by placental minces could result from the fact that the action of HCG is not direct and involves a long series of intracellular enzymatic reactions.

When placental minces are supplemented with NADP plus 3′,5′-AMP, there is a slight increase in the formation of estrogens. This would tend to suggest that 3′,5′-AMP could be one of the factors in the chain of events leading to the increased estrogen formation by HCG. Such a possibility has been suggested by Haynes et al (285) to explain the mode of action of ACTH in the adrenal cortex and by Marsh et al (286) to explain the mode of action of LH in the corpus luteum. 3′,5′-AMP does not promote an increase in estrogen formation from testosterone to the same extent as HCG. It has frequently been suggested that intact cell membranes are relatively impermeable to this compound (287). However, when HCG is present with NADP plus 3′,5′-AMP, the increase in estrogen formation is higher (73%) than with NADP plus HCG (59%).

The consistant higher production of 17β-hydroxy-steroids by HCG in the presence of NADP, NADP plus 3′,5′-AMP or NADP plus G-6-P, would suggest that another action of HCG could be to protect the 17β-hydroxyl group of the steroid molecule; thereby modifying the equilibrium of 17-hydroxyl $\rightleftarrows$ 17-ketone through an action on 17β-hydroxy-steroid dehydro--genase.

Chapter II

Placental homogenates

A) Effect of HCG on the metabolism of 4-^{14}C-testosterone by placental homogenates in the presence of various co-factors:

In chapter I all our studies were conducted with placental minces. In order to explore the possibility that cellular integrity is reuired for the action of HCG on placental aromatization, as was reported for ACTH in the instance of the adrenal cortex (288,289), investigations were carried out with placental homogenates.

Experimental conditions

Preincubated minces were used for the preparation of the homogenates as has been described in methods (page 45). An aliquot of the homogenate cont--aining an equivalent of 500 mg of tissue per 6 ml of KRBG buffer was used for each incubation. The precursor being 4-^{14}C-testosterone (1,776,700 d.p.m., 3.9 µg). Two experiments were run in parallel the same day.

Experiment 1: consisted of 6 incubation flasks, incubated for 2 hours;

flask 1. control,

flask 2. HCG (300 I.U.).

flask 3. NADP (2.6 µmol).

flask 4. NADP (2.6 µmol), HCG (3000 I.U.).

flask 5. NADP (2.6 µmol), 3′,5′-AMP (30.4 µmol).

flask 6. NADP (2.6 µmol), 3′,5′-AMP (30.4 µmol), HCG (3000 I.U.)

Experiment 2: consisted of 5 incubation flasks, incubated for 5 minutes;

flask 1. control.

flask 2. NADP (2.6 µmol), G-6-P (8.2 µmol).

flask 3. NADP (2.6 μmol), G-6-P (8.2 μmol), HCG (3000 I.U.)

flask 4. NADP (2.6 μmol), G-6-P (8.2 μmol), 3′,5′-AMP (30.4 μmol).

flask 5. NADP (2.6 μmol), G-6-P (8.2 μmol), 3′,5′-AMP (30.4 μmol), HCG (3000 I.U.).

Results and discussion

In our studies with minces, in the presence of NADP plus G-6-P, the quantity of precursor added was used up within 20 minutes. In order to have an excess substrate at the end of the incubation period, not only the weight of the tissue per incubation was reduced but in experiment 2 the time of incubat--ion was restricted to 5 minutes.

Table XII shows the percentage conversion from the 4-^{14}C-testosterone to various steroids when the incubations were carried out for 2 hours. Besides androstenedione, estrone, estradiol and 19-hydroxy-androstenedione as in placental minces, 19-hydroxy-testosterone was also detected in some cases. This compound has been very often detected in studies with placental homogenates. Due to the unavailability of ^{3}H-19-hydroxy-testosterone, the experimental losses of this compound could be corrected only with respect to the losses established for estrone, estradiol, testosterone or androstenedione, which were processed simultaneously.

In the control, the only conversion product obtained in placental homogenates was ^{14}C-androstenedione (11.7%). The conversion of testosterone to androstenedione was decreased to 5.0% when HCG was present. In the presence of NADP, estrone was the major conversion product obtained from 4-^{14}C-testo--sterone (88.5%). There was a small conversion to androstenedione (1.5%) and to estradiol (1.2%).

It is interesting to note the absence of 19-hydroxy-androstenedione production by placental homogenates incubated with NADP, as under the same

TABLE XII

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY PLACENTAL HOMOGENATES IN THE PRESENCE OF VARIOUS CO-FACTORS

	% Conversion from ^{14}C-testosterone to:					
	*TESTO	ASD	E_1	E_2	19-OH-ASD	19-OH-TESTO
Control	78.1	11.7	-	-	-	-
HCG	83.6	5.0	-	-	-	-
NADP	1.3	1.5	88.5	1.2	-	-
NADP + HCG	2.2	0.5	47.9	36.6	0.6	1.0
NADP + 3′,5′-AMP	0.9	1.0	78.9	3.3	0.5	-
NADP + 3′,5′-AMP + HCG	3.8	0.3	45.4	42.5	-	2.9

* Unconverted substrate recovered.

experimental conditions, this steroid was isolated in significant amounts when incubations were carried out with placental minces (chpter 1, C-(ii).). When HCG was added simultaneously with NADP, there was a decrease not only of androstenedione (0.5%), but of estrone (48%) as well,which was compensated by an increase of estradiol (36.6%). There were also small conversions to 19--hydroxy-androstenedione (0.6%) and 19-hydroxy-testosterone (1.0%). When 3′,5′-AMP was added with NADP, the percentage yield to estrone (79%) was slightly decreased with a simultaneous increase in the formation of estradiol (3.3%). However, the addition of HCG to NADP plus 3′,5′-AMP resulted in a very significant shift in the formation of estrone to estradiol; i.e., there was a decrease of estrone (45.4%) and a significant increase of estradiol (from 3.3% to 42.5%). There was also a small conversion to 19-hydroxy-testosterone (2.9%).

In experiment 1, although the weight of tissue incubated was half the weight of the tissue used in studies with placental minces, only in the control was there an excess of substrate left at the end of the 2 hours incu--bation. For this reason, it is difficult to draw a conclusion on the effect of HCG and/or 3′, 5′-AMP on placental aromatization.

The results of experiment 2, expressed as percentage conversion from the precursor are presented in Table XIII. Once again the only conversion pro--duct obtained in the control was androstenedione (4.4%). In the presence of NADP plus G-6-P. only 8.4% of ^{14}C-precursor remained unconverted at the end of 5 minutes of incubation. This suggests that testosterone is very rapidly con--verted in placental homogenates in the presence of an NADPH generating system. Under these conditions, there was a significant radioactivity incorporated in androstenedione (31.3%), estrone (28%) and estradiol (12%). There was also a small incorporation of radioactivity in 19-hydroxy-androstenedione (2.6%) and 19-hydroxy-testosterone (0.4%). When HCG was added to the incubation media in the presence of NADP plus G-6-P, 4-^{14}C-testosterone was not converted to the

TABLE XIII

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY PLACENTAL HOMOGENATES IN THE PRESENCE OF VARIOUS CO-FACTORS

	% Conversion from ^{14}C-testosterone to:					
	*TESTO	ASD	E_1	E_2	19-OH-ASD	19-OH-TESTO
Control	83.6	4.4	-	-	-	-
NADP + G-6-P	8.4	31.3	28.0	11.9	2.6	0.4
NADP + G-6-P + HCG	50.6	3.1	6.1	31.5	0.7	2.3
NADP + G-6-P + 3′,5′-AMP	41.0	8.0	6.7	32.9	1.0	2.1
NADP + G-6-P + 3′,5′-AMP + HCG	44.4	0.7	3.7	31.2	-	2.8

* Unconverted substrate recovered.

same extent (50.6% left) to androstenedione (3.1%), estrone (6.1%) and 19--hydroxy-androstenedione (0.7%). However, there was an increase in the form--ation of estradiol (31.5%) and 19-hydroxy-testosterone (2.3%).

The effect of 3′,5′-AMP in the presence of NADP plus G-6-P on the percentage conversion of ^{14}C-testosterone to estrone (6.7%), estradiol (32.9%), 19-hydroxy-androstenedione (1.0%) and 19-hydroxy-testosterone (2.1%) was very similar to the effect of HCG in the presence of NADP plus G-6-P. The only effect of the simultaneous addition of HCG to NADP plus G-6-P plus 3′,5′-AMP, was a decreased formation of androstenedione (0.7%) and estrone (3.7%).

The percentage conversion of testosterone to 17β-hydroxy-steroids (testosterone plus estradiol plus 19-hydroxy-testosterone) as well as to steroids possessing an aromatic ring A (estrone plus estradiol) were calculated from Tables XII and XIII. The graphic representation of these values are shown in Figures 9 and 10 respectively. When HCG was present, regardless of the co--factors or the length of an incubation, its effect was to increase the pro--duction of steroids possessing a 17β-hydroxyl group. This increase was a consequence of either an increase in the production of estradiol and 19-hydroxy--testosterone or a decrease in the utilization of testosterone. However, when 3′,5′-AMP was present besides NADP plus G-6-P, HCG did not increase the forma--tion of 17β-hydroxy-steroids. In the same way it can be seen in Table XIII, that the conversion to estrone, estradiol, 19-hydroxy-androstenedione and 19--hydroxy-testosterone were very similar in the presence of NADP plus G-6-P plus HCG, or NADP plus G-6-P plus 3′,5′-AMP. This would suggest that 3′,5′-AMP acts like HCG in placental homogenates when NADP plus G-6-P are present and that the action of HCG and 3′,5′-AMP on 17β-hydroxy-steroids is not cumulative.

Figures 9 and 10 also indicate the total conversions to estrone plus estradiol. It can be seen that there was no significant change in the aromati--zation products under the action of HCG. This is important, since in the

FIGURE 9

GRAPHIC REPRESENTATION OF THE ACTION OF HCG ON 17β-HYDROXY-STEROIDS (TESTO--STERONE PLUS ESTRADIOL PLUS 19-HYDROXY-TESTOSTERONE) AND AROMATIZATION PRODUCTS (ESTRONE PLUS ESTRADIOL); PLACENTAL HOMOGENATES INCUBATED IN THE PRESENCE OF NADP OR NADP+3',5'-AMP FOR 120 MINUTES.

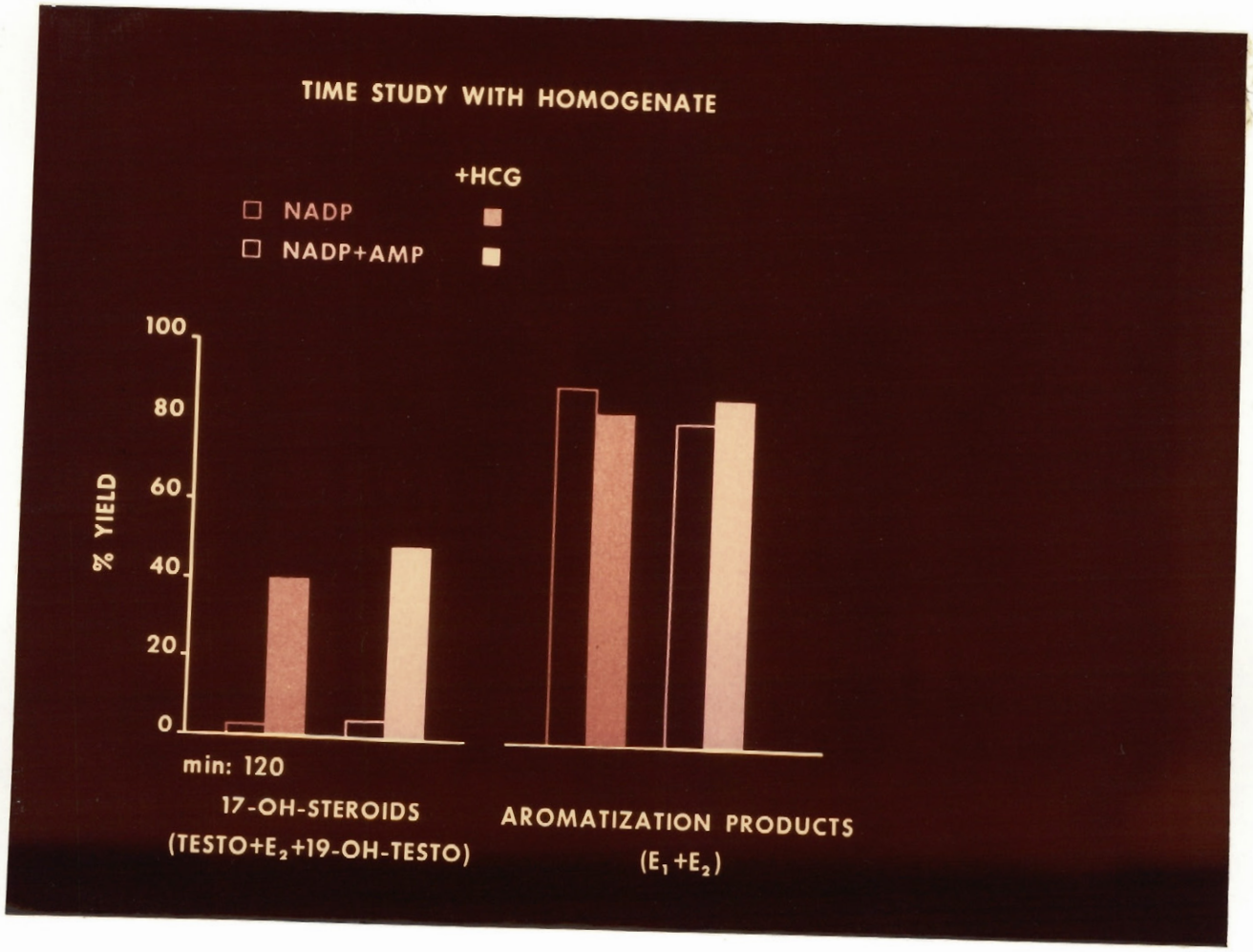
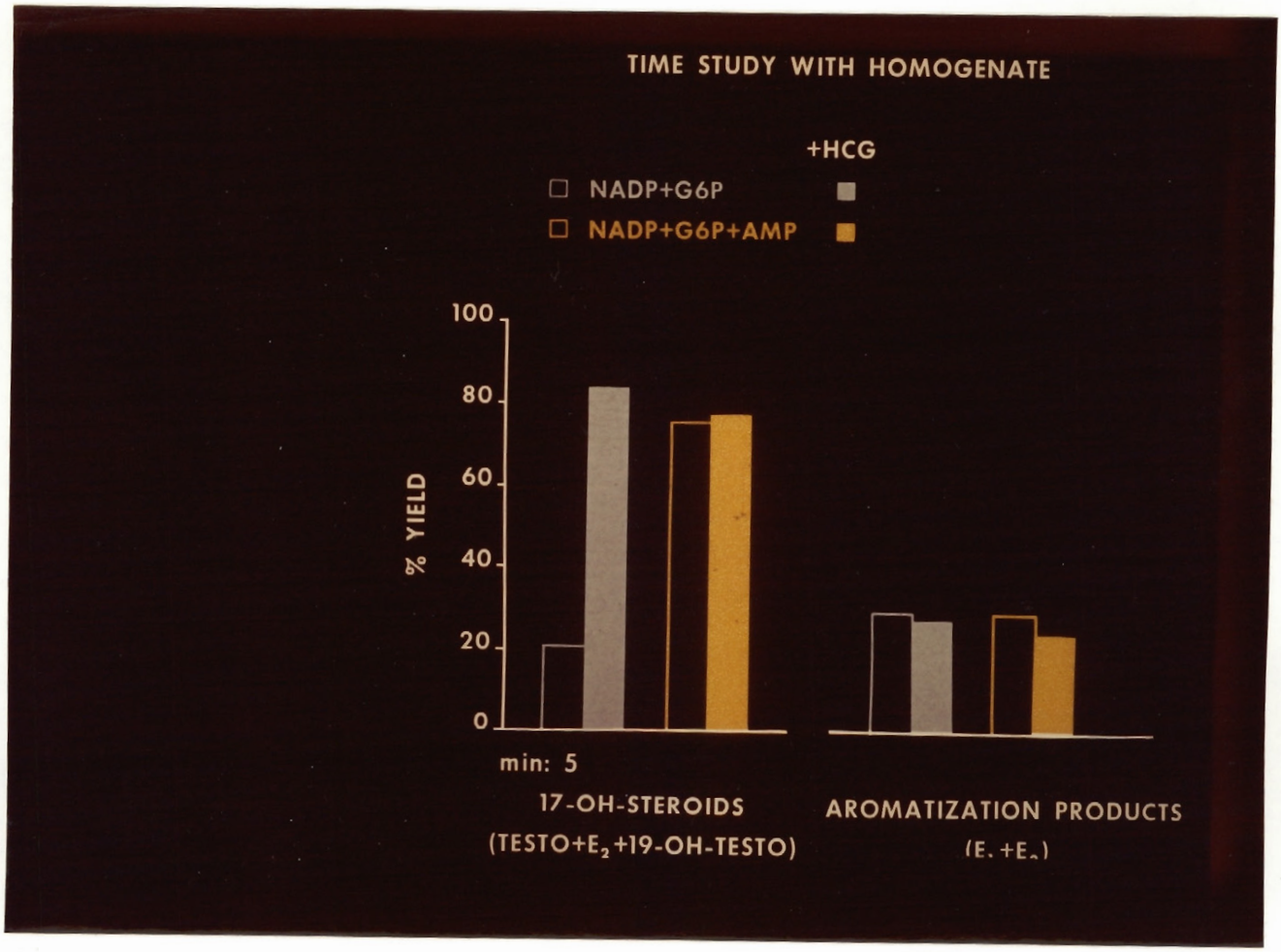


FIGURE 10

GRAPHIC REPRESENTATION OF THE ACTION OF HCG ON 17β-HYDROXY-STEROIDS (TESTO--STERONE PLUS ESTRADIOL PLUS 19-HYDROXY-TESTOSTERONE) AND AROMATIZATION PRODUCTS (ESTRONE PLUS ESTRADIOL); PLACENTAL HOMOGENATES INCUBATED IN THE PRESENCE OF NADP PLUS G-6-P OR NADP PLUS G-6-P PLUS 3′,5′-AMP FOR 5 MINUTES.



studies with placental minces, there was an increase in the formation of estrone plus estradiol with HCG in the presence of either NADP or NADP plus 3',5'-AMP in incubations carried out for 2 and 4 hours. However, in experiment 1, there was already a maximal conversion to estrone plus estradiol with NADP (90%) or NADP plus 3',5'-AMP (82%), so that the action of HCG, if positive, could not be detected. In experiment 2, the incubations were too short (5 min--utes only) to draw any conclusion with regard to a lag of action of HCG on aromatization (chapter I, (D).).

The conversions of testosterone to estrone and estradiol as defined by the ratio estrone/estradiol are shown in Table XIV. It can be seen that regardless of the co-factor present or the time of incubation, the effect of HCG was to decrease the estrone/estradiol ratios in minces as well as in homogenates. This decrease seemed even more pronounced in incubations with homogenates.

B) Effect of HCG on the metabolism of 4-^{14}C-testosterone by placental homogenates incubated at different time intervals:

In placental minces, time studies indicated that there was a lag period in the action of HCG (chapter I, (D).). Furthermore, HCG resulted in the increased formation of estrone plus estradiol in the presence of NADP and NADP plus 3',5'-AMP (chapter I,C-(ii).).

In incubation of placental homogenates (chapter II, (A).), the reaction of HCG on the aromatization of testosterone could not be evaluated since in experiment 1, the substrate concentration was too low and in experi--ment 2, a longer incubation period would have been required. To further investigate the action of HCG, a time incubation study was conducted with placental homogenates.

TABLE XIV

RATIOS OF ESTRONE OVER ESTRADIOL-17β OBTAINED FROM PLACENTAL INCUBATION STUDIES WITH 4-^{14}C-TESTOSTERONE

	E_1 / E_2 ratios	
	Minces*	Homogenates*
Control	7.6	-
NADP	10.5	73.7
NADP + HCG	5.8	1.3
NADP + 3′,5′-AMP	6.1	23.9
NADP + 3′,5′-AMP + HCG	3.3	1.1
NADP + G-6-P	9.8	2.4**
NADP + G-6-P + HCG	3.5	0.2
NADP + G-6-P + 3′,5′-AMP	-	0.2
NADP + G-6-P + 3′,5′-AMP + HCG	-	0.1

* Incubations were carried out for 2 hours

** Incubations were carried out for 5 mins.

Experimental conditions

Each incubation flask contained an aliquot of placental homogenate equivalent to 250 mg of tissue per 3 ml of KRBG. Five sets of incubations were carried out at the same time. Each set of incubation consisted of 5 incubation flasks.

flask 1. control.

flask 2. NADP (2.6 μmol).

flask 3. NADP (2.6 μmol), HCG (3000 I.U.).

flask 4. NADP (2.6 μmol), G-6-P (8.2 μmol).

flask 5. NADP (2.6 μmol), G-6-P (8.2 μmol), HCG (3000 I.U.).

All the flasks were incubated with 4-^{14}C-testosterone. In order to have an excess of precursor, 39.87 μg of non-radioactive testosterone were added. Thus, each incubation flask contained 2,040,000 d.p.m., and 44.4 μg of 4-^{14}C-testosterone. Flasks 1,2,3,4 and 5 were incubated for 5,20,60,120,and 240 minutes respectively under the conditions already described in methods.

Results and discussion

The percentage conversion of 4-^{14}C-testosterone to various steroids at different times of incubations is indicated in Table XV. The graphic representations of the percentage conversions of 4-^{14}C-testosterone to each steroid at different times of incubation are plotted and shown in Figures 11, 12, 13 and 14.

In the control, the only conversion product obtained was 4-^{14}C-andro--stenedione; the greatest conversion value was 3.0% after 4 hours of incubation, 74-76% of the testosterone added was recovered as such. When NADP or NADP plus G-6-P were present in the incubation medium, the precursor was almost completely utilized after 2 hours (Figure 11). The utilization of the precursor was dependant on the co-factors used and increased gradually from 5 minutes up to 4 hours of incubation. Addition of HCG with either NADP or NADP plus G-6-P,

TABLE XV

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY PLACENTAL HOMOGENATES INCUBATED AT DIFFERENT TIME INTERVALS

Time incubated		%-Conversion from ^{14}-C-testosterone to:					
		*TESTO	ASD	E_1	E_2	19-OH-ASD	19-OH-TESTO
5 min	Control	74.8	0.6	-	-	-	-
	NADP	51.2	24.0	0.7	1.3	0.4	-
	NADP + HCG	74.1	2.0	0.6	1.8	-	-
	NADP + G-6-P	60.8	12.8	0.8	3.0	-	-
	NADP + G-6-P + HCG	69.6	1.5	0.6	3.6	-	-
20 min	Control	76.3	0.8	-	-	-	-
	NADP	20.0	42.3	2.0	1.5	0.4	-
	NADP + HCG	63.4	4.9	0.8	3.2	-	-
	NADP + G-6-P	40.6	18.9	2.4	5.2	-	-
	NADP + G-6-P + HCG	60.9	1.9	1.0	6.5	-	-
60 min	Control	73.7	1.0	-	-	-	-
	NADP	5.7	63.0	4.2	1.7	1.0	-
	NADP + HCG	62.5	7.9	2.2	3.4	0.5	0.7
	NADP + G-6-P	24.5	30.8	6.2	7.1	0.9	-
	NADP + G-6-P + HCG	55.0	3.7	2.6	10.0	-	1.1
120 min	Control	74.6	1.9	-	-	-	-
	NADP	3.7	59.9	8.2	1.8	2.0	-
	NADP + HCG	60.9	9.8	5.7	3.5	0.5	0.9
	NADP + G-6-P	4.7	52.1	11.4	3.2	1.7	-
	NADP + G-6-P + HCG	53.5	4.7	5.8	9.6	0.5	1.7
240 min	Control	73.9	2.8	-	-	-	-
	NADP	3.0	60.1	8.6	1.9	1.9	-
	NADP + HCG	54.3	14.9	6.1	3.7	1.3	1.4
	NADP + G-6-P	4.5	52.6	12.7	2.9	1.9	-
	NADP + G-6-P + HCG	53.9	5.7	6.1	9.2	0.7	1.5

* Unconverted substrate recovered.

FIGURE 11

CONCENTRATIONS OF UNCONVERTED TESTOSTERONE RECOVERED UNDER THE ACTION OF HCG; PLACENTAL HOMOGENATES INCUBATED IN THE PRESENCE OF NADP OR NADP PLUS G-6-P FOR DIFFERENT LENGTHS OF TIME.

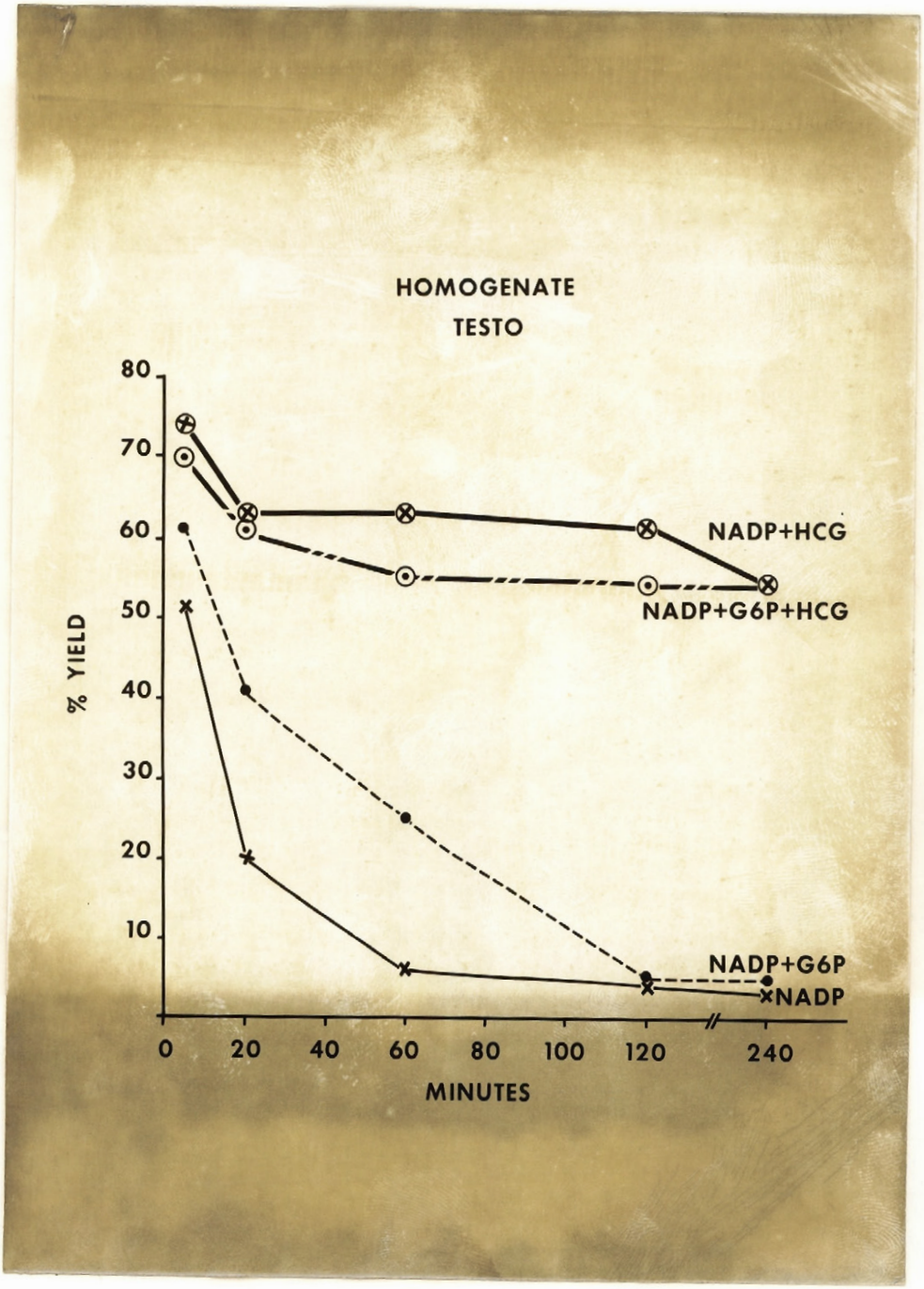


FIGURE 12

EFFECT OF HCG ON THE PERCENTAGE CONVERSION OF TESTOSTERONE TO ANDROSTENEDIONE BY PLACENTAL HOMOGENATES INCUBATED IN THE PRESENCE OF NADP OR NADP PLUS G-6-P FOR DIFFERENT LENGTHS OF TIME.

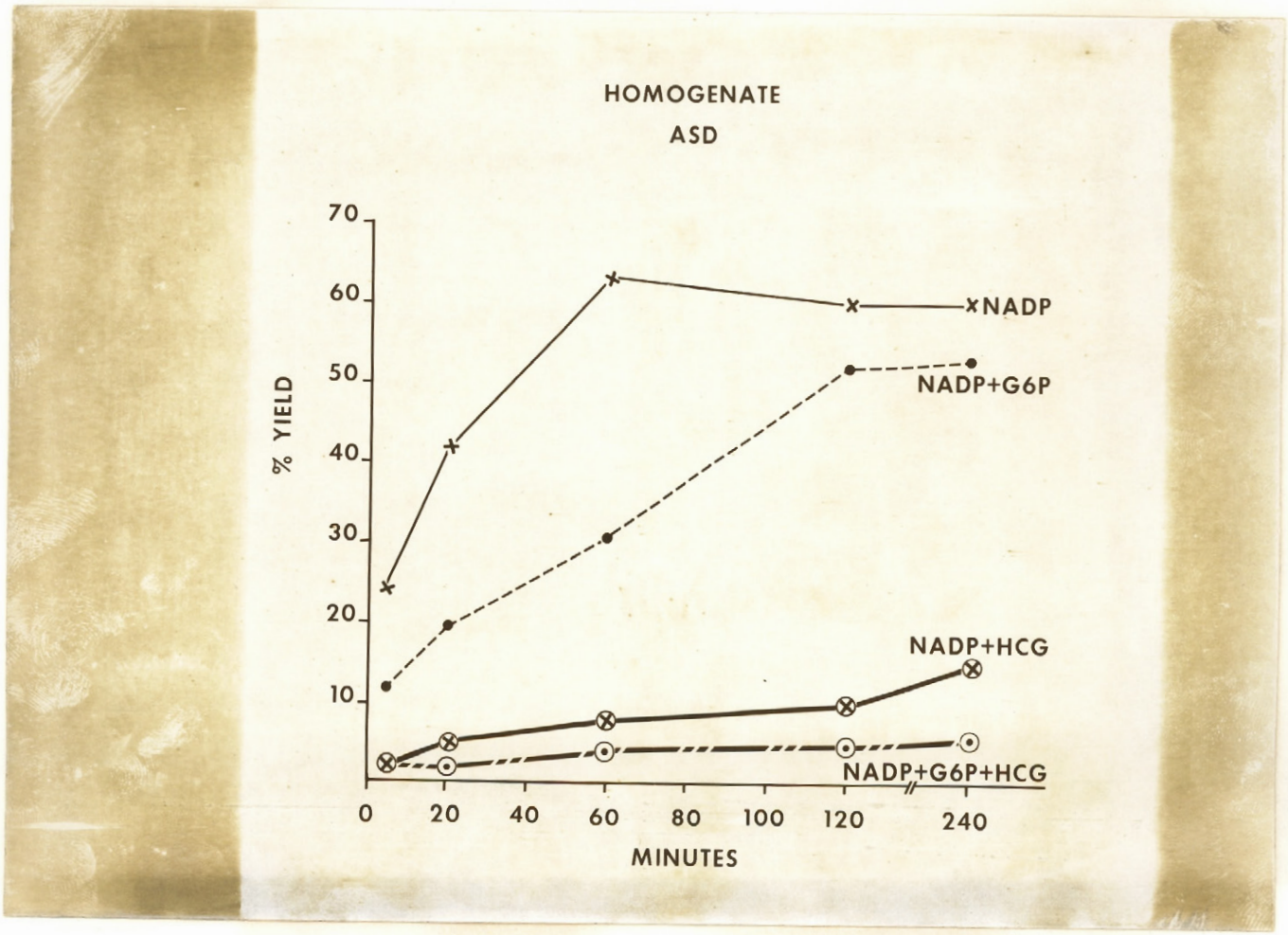


FIGURE 13

EFFECT OF HCG ON THE PERCENTAGE CONVERSION OF TESTOSTERONE TO ESTRONE BY PLACENTAL HOMOGENATES INCUBATED IN THE PRESENCE OF NADP OR NADP PLUS G-6-P FOR DIFFERENT LENGTHS OF TIME.

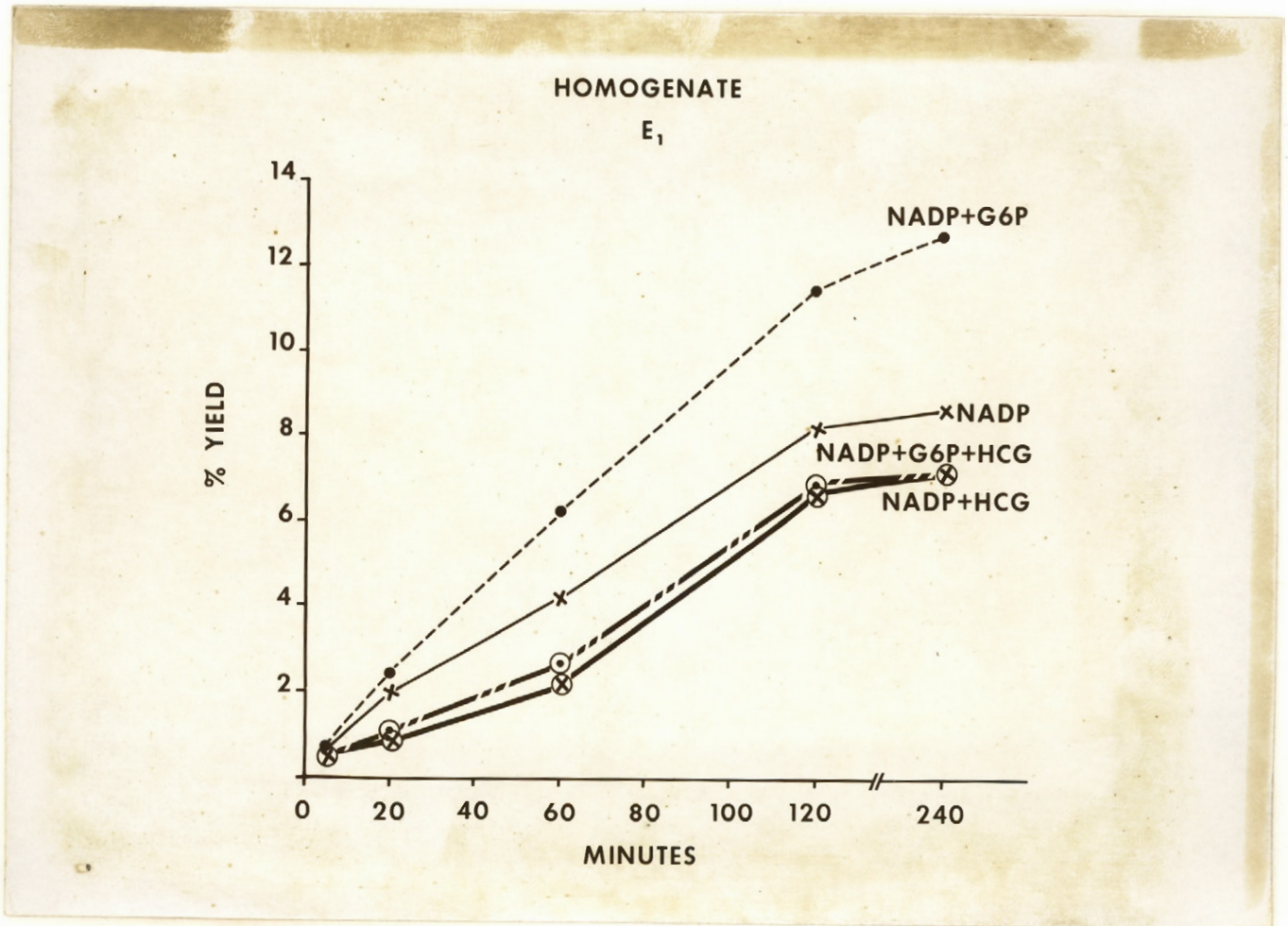
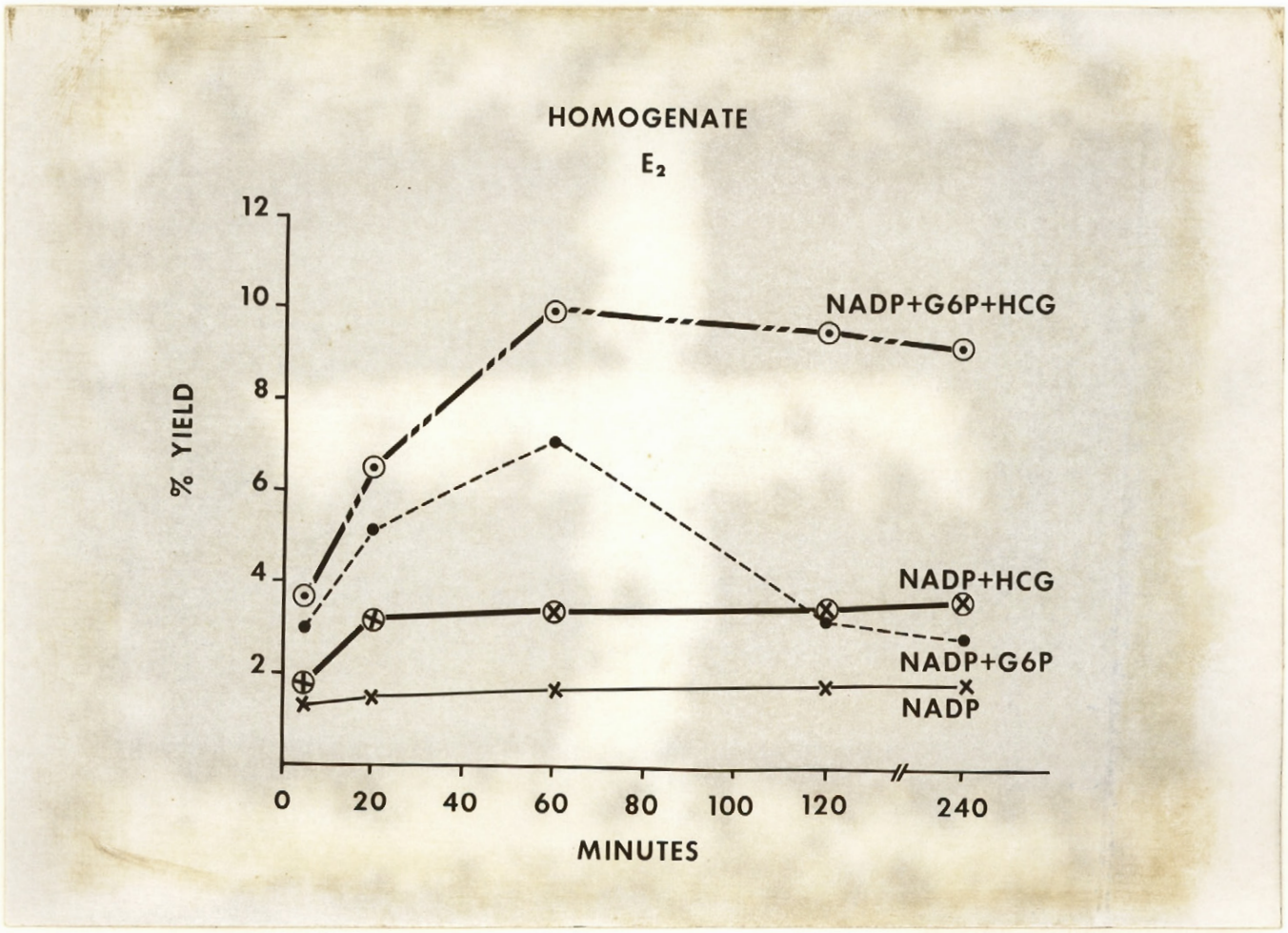


FIGURE 14

EFFECT OF HCG ON THE PERCENTAGE CONVERSION OF TESTOSTERONE TO ESTRADIOL BY PLACENTAL HOMOGENATES INCUBATED IN THE PRESENCE OF NADP OR NADP PLUS G-6-P FOR DIFFERENT LENGTHS OF TIME.



resulted in a lesser degree of utilization of testosterone. At the end of 4 hours of incubation 54% of the precursor remained unconverted.

The conversion of testosterone to androstenedione in the presence of NADP was increased as the time of incubation was prolonged (Figure 12). It reached the value of 63% at 60 minutes and thereafter levelled on. When HCG was added with NADP, a very significant decrease in the formation of ^{14}C-and--rostenedione was observed. When G-6-P was added simultaneously with NADP to the incubate of the homogenate, the maximum accumulation of androstenedione was obtained after 2 hours (52%). This value was slightly lower that with NADP alone (60%). Supplementation of HCG to the NADPH generating system resulted in a greater decrease in the conversion to ^{14}C-androstenedione than when HCG was used in combination with NADP (Figure 12).

Figure 13 graphically represents the values of estrone in these time studies. There was no conversion of testosterone to estrone in the controls. Addition of NADP to homogenates gradually increased the conversion to estrone up to 4 hours. G-6-P when added with NADP, increased the percentage conversion to estrone which was always higher for each length of incubation than the values obtained with NADP alone. When HCG was added in combination with NADP or NADP plus G-6-P, there was a very marked decrease in the conversion to estrone; compared to the value obtained with NADP or NADP plus G-6-P. This was more apparent after 20 minutes onwards. The maximum conversion to estrone was 6.1%, only at 4 hours.

Estradiol was not formed in the absence of co-factors. In the presence of NADP, the conversions to estradiol were still small and increased very little with time (1.9% at 4 hours). Addition of HCG, increased these conversions at all times of incubation, but there a plateau after 20 minutes (Figure 14). In the presence of an NADPH generating system, the conversions to estradiol by placental homogenates were higher than with NADP alone. However, after 60 min--utes of incubation, there was a significant drop in the quantity of

estradiol formed (3.2%). Addition of HCG to the NADPH generating system, increased further the conversion to estradiol at all times of incubation and prevented the fall at 2 and 4 hours.

Small amounts of 19-hydroxy-androstenedione and 19-hydroxy-testo--sterone were measured in these studies. However, they increased very little with time. The formation of 19-hydroxy-testosterone was observed only in the presence of HCG (Table XV).

The sum of 17β -hydroxy-steroids (i.e., testosterone plus estradiol plus 19-hydroxy-testosterone) as well as the aromatization products (estrone plus estradiol) of ^{14}C-testosterone were calculated for placental homogenates as has been described for minces (chapter I). These values are recorded in Table XVI.

One can see that in all cases the presence of HCG in the incubation media increased the formation of 17β-hydroxy-steroids. This increase was apparent even when the values for testosterone were not included in computive, the values of total 17β- hydroxy-steroids. Aromatization products were not significantly increased by HCG; even when the incubations were prolonged up to 4 hours, although an excess substrate (either as testosterone or as androstene--dione) was always available. This was in contrast to the effect observed in minces supplemented with NADP or NADP plus 3',5'-AMP (chapter I).

Discussion

The conversion of testosterone to estrogens can also be demonstrated in placental homogenates. However, for this reaction to take place it was necessary to supplement the systems with co-factors; since in their absence, the only conversion product obtained was ^{14}C-androstenedione. When the homogenates were supplemented with NADP, NADP plus 3',5'-AMP or NADP plus G-6-P, the conversions to estrogens were much higher than with minces and this

TABLE XVI

EFFECT OF HCG ON TOTAL 17β -OH-STEROIDS AND AROMATIZATION PRODUCTS FROM 4-^{14}C-TESTOSTERONE BY PLACENTAL HOMOGENATES INCUBATED AT DIFFERENT TIME INTERVALS

Time Incubated	Additives	%-Conversion of ^{14}C-precursor to: 17β -OH-Steroids (testo + E_2 + 19-OH-testo)	Aromatization products (E_1 + E_2)
5 min	NADP	52.5	2.0
	NADP + HCG	75.9	2.4
	NADP + G-6-P	63.8	3.8
	NADP + G-6-P + HCG	73.2	4.2
20 min	NADP	21.5	3.5
	NADP + HCG	66.6	4.0
	NADP + G-6-P	45.8	7.6
	NADP + G-6-P + HCG	67.4	7.5
60 min	NADP	7.4	5.9
	NADP + HCG	66.6	5.7
	NADP + G-6-P	31.6	13.3
	NADP + G-6-P + HCG	66.1	12.6
120 min	NADP	5.5	10.0
	NADP + HCG	65.3	9.2
	NADP + G-6-P	7.9	14.6
	NADP + G-6-P + HCG	64.8	15.4
240 min	NADP	4.9	10.5
	NADP + HCG	59.4	9.8
	NADP + G-6-P	7.4	15.6
	NADP + G-6-P + HCG	64.6	15.3

in spite of the fact that the weight of tissue per incubation was half of that used in incubation of minces.

It was interesting to observe that HCG did not increase the total aromatization products (estrone plus estradiol) in placental homogenates, while a very significant increase was observed in placental minces. In the case of ACTH, it is known that when cellular organization is disrupted, there is a lack of response to the stimulatory effect of ACTH on the adrenal cortex, although corticosteroids can be synthesized in such systems (288,290-292). Our findings would suggest that the integrity of the cellular membrane is necessary for the stimulatory action of HCG on the synthesis of estrogens in the placenta.

However, the total production of 17β-hydroxy-steroids was signifi--cantly increased in placental homogenates in the presence of HCG. The increase in 17β-hydroxy-steroids with HCG was very much more pronounced in placental homogenates than in placental minces, This was due (i) mainly, to an increased formation of estradiol which resulted in a simultaneous decrease in the formation of estrone, (ii) to an increase in the formation of 19--hydroxy-testosterone, (iii) or to a decreased utilization of ^{14}C-testosterone. This suggests that one of the actions of HCG may be to protect the 17β--hydroxyl group of the steroid molecule.

Chapter III

Placental subcellular fractions

Action of HCG on the metabolism of 4-^{14}C-testosterone by various subcellular fractions of the term placenta:

The chief purpose of this study was to see if HCG had any action on the aromatizing systems at the subcellular level. In 1959, Ryan demonstrated that the enzyme systems necessary for the aromatization of ring A of C-19--steroids are located in the microsomal fraction of the human placenta, but was unable to demonstrate their presence in the mitochondrial fraction (86). He also showed that NADPH and molecular oxygen were essential for aromatization (86). Among subsequent investigators (293-299), only Shaw et al (300) in 1969, have reported estrogen synthesis from testosterone, not only by the microsomal fraction of the human term placenta, but also by the mitochondrial fraction and this in relatively high yield.

Experimental conditions

The subcellular fractions were prepared as described in methods (page 45-46). Each incubation flask contained the homogenate or subcellular fraction equivalent to 1 gm of placental tissue in a total volume of 6 ml (3.3 ml of 0.25 M sucrose plus 2.7 ml of KRBG buffer). Four sets of incubations were prepared. Each set consisted of 8 incubation flasks:

flask 1. one gram of whole homogenate.

flask 2. the reconstitution of mitochondrial plus microsomal plus supernatant fractions.

flask 3. mitochondrial fraction only.

flask 4. microsomal fraction only.

flask 5. supernatant fraction only.

flask 6. mitochondrial plus microsomal fractions .

flask 7. mitochondrial plus supernatant fractions.

flask 8. microsomal plus supernatant fractions.

Set 1, was supplemented with NADP; set 2, with NADP plus HCG; set 3, with NADP plus G-6-P and set 4, with NADP plus G-6-P plus HCG. All flasks contained G-6-P-dehydrogenase (5 korngsberg units / incubation). The incubations were carried out with 4-^{14}C-testosterone (2,040,100 d.p.m., 4.5 μg) for a period of 2 hours under the usual conditions of incubation.

Results and discussion

Tables XVII and XVIII show the percentage conversion of 4-^{14}C--testosterone to various steroids by homogenates and different subcellular fractions in the presence of the various combination of co-factors.

One can see in Table XVII, that when the whole homogenate was incubated in the presence of NADP, the results are comparable to those already reported in chapter II, (A) , Table XII. Testosterone was converted almost completely to androstenedione (1.5%), estrone (72.6), estradiol (6.0%) and 19-hydroxy-androstenedione (0.5%). The presence of HCG with NADP resulted in a decreased formation of estrone (36%), which was compensated by an increase in the formation of estradiol (40.5%). The conversions to androstenedione and 19-hydroxy-androstenedione were not affected to a significant extent; however, 19-hydroxy-testosterone (0.6%) was detected. The resultsobtained with the pooled mitochondrial plus microsomal plus supernatant fractions incubated with NADP were similar to those obtained with the homogenate. However, the shift in the formation of estrone (20%) to estradiol (61.2%) observed with HCG was more pronounced than the one observed under the same conditions in the homogenate (estrone: 36%, estradiol: 40.5%).

TABLE XVII

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY VARIOUS SUBCELLULAR FRACTIONS AND HOMOGENATES OF TERM PLACENTA IN THE PRESENCE OF NADP

	%-Conversion from ^{14}C-testosterone to: NADP						NADP + HCG					
	*TESTO	ASD	E_1	E_2	19-OH--ASD	19-OH--TESTO	*TESTO	ASD	E_1	E_2	19-OH--ASD	19-OH--TESTO
HOMOGENATE	2.8	1.5	72.6	6.0	0.5	-	4.0	1.2	36.1	40.5	0.3	0.6
Mito + Micro + Super	2.7	1.2	73.2	6.8	0.5	-	4.8	0.5	19.9	61.2	0.5	4.0
Mitochondria	1.0	71.8	3.2	1.0	5.1	-	78.9	2.8	1.2	0.4	0.2	1.3
Microsomes	2.0	61.7	11.5	1.5	4.2	-	78.0	3.2	1.0	0.7	0.4	2.9
Supernatant	14.8	58.9	2.6	2.1	0.3	-	79.5	3.4	1.2	0.3	0.1	0.2
Mito + Micro	1.1	65.2	3.4	0.5	5.9	-	74.0	7.7	1.2	0.8	1.1	3.0
Mito + Super	2.7	0.8	15.9	66.0	0.4	-	44.7	3.0	18.3	12.7	1.0	5.3
Micro + Super	6.8	0.7	25.9	45.8	0.4	-	28.3	2.7	27.6	18.7	0.6	5.7

Mito — Mitochondria, Micro — Microsomes, Super — Supernatant.

* Unconverted substrate recovered.

TABLE XVIII

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY VARIOUS SUBCELLULAR FRACTIONS AND HOMOGENATES OF TERM PLACENTA IN THE PRESENCE OF NADP + G-6-P

	%-Conversion from ^{14}C-testosterone to:											
	NADP + G-6-P						NADP + G-6-P + HCG					
	*TESTO	ASD	E_1	E_2	19-OH--ASD	19-OH--TESTO	*TESTO	ASD	E_1	E_2	19-OH--ASD	19-OH--TESTO
Homogenates	7.2	1.2	74.3	3.5	0.3	0.7	0.9	0.6	58.5	21.2	-	2.2
Mito + Micro + Super	1.2	0.7	78.5	5.4	-	1.6	1.3	0.1	5.3	76.9	-	2.5
Mitochondria	9.4	0.6	30.2	48.3	0.5	1.1	3.7	0.1	2.6	68.0	-	6.3
Microsomes	7.4	0.3	24.9	54.1	0.3	1.5	4.6	0.1	1.0	74.9	-	3.9
Supernatant	48.4	21.6	2.2	8.6	0.4	0.5	77.7	0.6	0.2	6.7	-	0.5
Mito + Micro	1.7	0.7	83.6	3.7	-	0.4	2.3	0.2	10.5	67.2	-	2.7
Mito + Super	2.5	0.3	5.8	76.5	0.3	1.5	0.8	0.1	1.0	81.1	-	3.5
Micro + Super	3.5	0.3	12.7	65.7	0.5	1.9	0.9	0.1	1.1	73.5	-	2.7

Mito — Mitochondria, Micro — Microsomes, Super — Supernatant.

* Unconverted substrate recovered.

When either the mitochondrial or the microsomal or the supernatant fractions were incubated alone in the presence of NADP, there was a large accumulation of androstenedione (59-72%). The accumulation of 19-hydroxy--androstenedione (4.2-5.1%) observed in the mitochondrial or the microsomal fractions was greater than when similar studies were carried out with the homogenates or the pooled mitochondrial plus microsomal plus supernatant fractions. This increase in androstenedione and 19-hydroxy-androstenedione was accompanied by a simultaneous decrease in the formation of estrone (2.6--11.5%) and to estradiol (1.0-2.1%). If HCG was added besides NADP to the mitochondrial, microsomal or supernatant fractions, the utilization of the precursor was inhibited and there was an overall decrease in the formation of androstenedione (2.8-3.4%), estrone (1.0-1.2%), estradiol (0.3-0.7%) and 19-hydroxy-androstenedione (0.1-0.4%). However, under such conditions 19--hydroxy-testosterone was isolated.

When both mitochondrial plus microsomal fractions were combined and incubated in the presence of NADP, the conversion of testosterone to estrone, estradiol or 19-hydroxy-androstenedione didnot add up to the con--versions obtained in each fraction separately. Addition of HCG to NADP in the incubation flask containing the combined mitochondrial plus microsomal fractions produced a very marked decrease in the conversion of testosterone not only to estrone (1.2%) and 19-hydroxy-androstenedione (1.1%) but also to androstenedione (7.7%), whereas HCG promoted the formation of 19-hydroxy--testosterone (3.0%).

In the incubation media with NADP, when the supernatant fraction was added either to the mitochondrial or to the microsomal fractions, there was an increase in the production of estrone (16-26%) and estradiol (46-66%). Under such conditions the increased production of these two steroids was very

much greater than that expected from the sum of their production in the separate incubations of the supernatant and of the microsomal fractions or of the supernatant and of the mitochondrial fractions. This held true for other steroids such as, androstenedione or 19-hydroxy-androstenedione. More--over, when the supernatant fraction was combined with the mitochondrial fraction, the conversion of testosterone to estradiol (66%) was higher, and to estrone was smaller (16%) than when the supernatant was combined with the microsomal fraction (estradiol: 46% and estrone: 26%). If HCG was supple--mented with NADP in the incubation media of the supernatant, combined with either the mitochondrial or microsomal fraction, it did not affect the con--versions to estrone (18 and 28% respectively), whereas the conversions to estradiol were lower (12.7 and 18.7% respectively).

It must be pointed out here that when any two subcellular fractions (mitochondrial plus microsomal, mitochondrial plus supernatant or microsomal plus supernatant) were combined and incubated in the presence of HCG in a medium supplemented with NADP, the shift in the formation of estrone to estradiol was not observed.

Table XVIII shows the percentage conversion of testosterone to various steroids in the presence of either NADP plus G-6-P or NADP plus G-6-P plus HCG.

In the presence of NADP plus G-6-P, the conversion to different steroids by homogenates was comparable not only to that obtained in pooled mitochondrial plus microsomal plus supernatant fractions, but also to that obtained with NADP alone.

When mitochondrial or microsomal fractions were incubated separately, the simultaneous addition of G-6-P to NADP, increased the conversions to estrone (24.9-30.2%) and estradiol (48.3-54.1%) very markedly.

It is interesting to emphasize that a similar increase in estrone (16-26%) and estradiol (46-66%) was observed when either the mitochondrial or the microsomal fractions were incubated with the supernatant in the presence of NADP alone.

When the incubation media of the supernatant was supplemented with an NADPH generating system, there was a decreased utilization of the precursor, concomitant with a decrease in androstenedione (21.6%) formation and an increase in estradiol (8.6%) compared to the values obtained with NADP alone.

When the mitochondrial and microsomal fractions were combined and incubated in the presence of and NADPH generating system, there was a shift in the formation of estradiol (3.7%) to estrone (83.6%) compared to values obtained in incubations of the mitochondrial or the microsomal fractions separately. In contrast, this shift was in favour of estradiol when mitochondrial plus super--natant or microsomal plus supernatant were incubated under the same experimen--tal conditions.

The simultaneous addition of HCG, NADP and G-6-P in the homogenates, led once more to an increased formation of estradiol (21.2%) and to a decreased formation of estrone (58.5%) as was observed with NADP. The same phenomenon but more pronounced was observed in the pooled mitochondrial plus microsomal plus supernatant fractions (estradiol: 77%, estrone: 5.3%).

It must be pointed out that in the instance of all subcellular fractions (Table XVIII), with the exception of the supernatant, there was an increase in estradiol and 19-hydroxy-testosterone formation and a simultaneous decrease in estrone when HCG was added in the presence of an NADPH generating system. However, in the instance of the supernatant fraction, the utilization of testosterone was inhibited and its conversion to other steroids was decreased.

Since our interest was to observe the action of HCG on aromatizing

enzymes at the subcellular level, total aromatization products (estrone plus estradiol) were calculated as shown in Table XIX.

One can see that the conversions of testosterone to estrone plus estradiol by the homogenates (79%) or by the pooled mitochondrial plus micro--somal plus supernatant fractions (80-84%) in the presence of NADP or NADP plus G-6-P, were maximal, so that the action of HCG on aromatization (77-82%) could not be assessed.

When the mitochondrial or the microsomal or the supernatant fractions or the combination of mtiochondrial plus microsomal fractions were incubated in the presence of NADP, the conversions to estrone plus estradiol were signi--ficantly diminished (4-13%), compared to values obtained in the whole homogen--ate. However, addition of G-6-P or of the supernatant to either the mitochon--drial or to the microsomal fractions in the presence of NADP, increased the conversion to estrone plus estradiol very significantly. Whereas, the conversion to estrone plus estradiol (11%) did not increase to any significant extent; when the supernatant fraction was supplemented with NADP plus G-6-P.

Addition of HCG in the presence of NADP to any of the fractions (with the exception of the homogenate or pooled mitochondrial plus microsomal plus supernatant fractions), decreased aromatization compared to values obtained in the corresponding controls with NADP only.

Sine in the presence of NADP plus G-6-P, almost maximal conversions to estrone plus estradiol occured in the different fractions, the action of HCG was not observed.

Discussion

In these experiments, we were able to demonstrate the conversion of testosterone to androstenedione, estrone, estradiol, 19-hydroxy-androstenedione and 19-hydroxy-testosterone by various subcellular fractions of placenta in accordance with the results obtained with placental minces (chapter I) and

TABLE XIX

EFFECT OF HCG ON TOTAL AROMATIZATION PRODUCTS FROM 4-^{14}C-TESTOSTERONE BY VARIOUS SUBCELLULAR FRACTIONS AND HOMOGENATES OF TERM PLACENTA IN THE PRESENCE OF NADP OR NADP + G-6-P

	%-Conversion of ^{14}C-testosterone to total aromatization products ($E_1 + E_2$) in the presence of:			
	NADP	NADP + HCG	NADP + G-6-P	NADP+G-6-P+HCG
Homogenate	78.6	76.6	77.8	79.7
Mito + Micro + Super	80.0	81.8	83.9	82.2
Mitochondria	4.2	1.6	78.5	70.6
Microsomes	13.0	1.7	79.0	75.9
Supernatant	4.7	1.5	10.8	6.9
Mtio + Micro	3.9	2.0	87.3	77.7
Mito + Super	81.9	31.0	82.3	82.1
Micro + Super	71.7	46.3	78.4	74.6

Mito — Mitochondria, Micro — Microsomes, Super — Supernatant.

homogenates (chapter II).

It was demonstrated that not only the microsomal fraction but also the mitochondrial fraction contains the enzymatic systems necessary for the aromatization of C-19 steroids. As compared to this, the aromatizing activity in the supernatant fraction was negligible. As evidenced by the isolation of metabolites such as , 19-hydroxy-androstenedione and 19-hydroxy-testosterone, the 19-hydroxylase was also present in the mitochondrial and microsomal fractions. 19-Hydroxylase has been demonstrated to be associated with the mitochondrial fraction of the cells (288). However, Longchampt et al (293) isolated 19-hydroxy-androstenedione from incubations of human placental microsomes. A soluble 17β-hydroxysteroid dehydrogenase from human placenta has been isolated by various investigators (220).

In spite of our efforts to have a good separation of these different fractions, the isolation of a small aromatizing activity in the supernatant fraction indicates the contamination with the microsomal fraction. However, this contamination of one subcellular fraction by the other seems almost inevitable with our present day techniques for the separation of organelles by ultracentrifugation. An example of this situation is offered by the elegant studies of Halkerston et al (302) in which the incubation of 4-^{14}C-cholesterol with carefully isolated adrenal mitochondrial pellets, resulted in the detection of metabolites like cortisol;in whose elaboration microsomal 17α-and 21--hydroxylases are involved. However, the aromatizing activity in the mitochon--drial fraction was very significant and cannot be considered as a contamination by the microsomal fraction.

In the presence of NADP alone, there was very little aromatizing activity in the incubations of the mitochondrial or the microsomal fractions. Whereas when G-6-P was added to these incubations with NADP, the aromatizing activity was very significantly increased. This increased activity in the

mitochondrial or the microsomal fractions was almost duplicated when the supernatant fraction was substituted for G-6-P, in the incubation media. On the other hand, when the supernatant was supplemented with G-6-P and NADP, the aromatizing activity was still very small.

These observations partially confirm the findings of Ryan et al (86), who showed that the aromatizing enzymes are located in the microsomes, whereas the supernatant provides the elements necessary for the generation of NADPH. Our experiments however show that this aromatizing activity resides in the mitochondrial fraction as well. This confirms the observations of Shaw et al (300).

Incubations of mitochondrial fraction with NADP, gave a lower yield of estrone plus estradiol than that obtained with microsomal fraction under the same experimental conditions. In our experiments, the mitochondrial fraction was washed once, whereas the microsomal fraction was not subjected to any washing. Shaw et al (300) have shown that washing the mitochondrial or microsomal fractions, decreases enzymatic activities. In the present study, the decreased formation of estrone plus estradiol by mitochondrial fraction compared to microsomal fraction could be attributed to the washing of the mitochondrial fraction.

The action of HCG in the subcellular fractions is difficult to explain. As was seen in chapter II, (B), the addition of HCG to placental homogenates did not affect the extent of aromatization. Due to the fact that in the presence of NADP or NADP plus G-6-P, the aromatization was almost complete (78-84%) in homogenates or in pooled mitochondrial plus microsomal plus supernatant fractions, the action of HCG on aromatization was not apparent. The same applies to the action of HCG on estrone plus estradiol in various subcellular fractions, when they were already maximally stimulated with NADP plus G-6-P. However, when various subcellular fractions (with the exception of

pooled mitochondrial plus microsomal plus supernatant fractions) were incubated with NADP alone, the presence of HCG decreased the total conver--sions to estrone plus estradiol significantly.

From our observations with placental minces (chapter I), as well as from the findings of other investigators (111), it would seem that HCG could act by increasing the NADPH concentration in the placental cells. The mechanism by which greater amounts of NADPH are available likely implies the activation of phosphorylase by 3′,5′-AMP and the subsequent increase in G-6-P. Such a chain of reactions was reported in the adrenal gland to explain the mode of action of ACTH on the biosynthesis of corticosteroids (285). However, in cell--free systems; such as adrenal homogenates neither ACTH nor 3′,5′-AMP was found to stimulate the corticosteroid synthesis significantly. This is presu--mably due to a dilution or to a loss of activity of phosphorylase (303). In the studies conducted by us with placental homogenates (chapter II) and with subcellular fractions, there was no stimulation of aromatization with HCG. These findings suggest that for a stimulatory action of HCG to take place, an intact cell is required.

Since HCG cannot provide the increase in concentration of G-6-P in subcellular fractions, it may act on the aromatizing enzymes directly or it may limit the availability of NADPH or it may just act to protect the 17β--hydroxyl group of testosterone. The combination of any of these conditions would result in a decreased utilization of the precursor and a decrease in the formation of aromatization products as well as of other steroids. However, HCG seems to have a specific effect on the formation of 19-hydroxy-testosterone, the percentage conversions of this compound were always increased in different subcellular fractions under the action of HCG.

It must be pointed out here that, when different subcellular fractions (with the exception of the supernatant) were incubated in the presence of NADP

plus G-6-P, there was a maximal conversion to estrone plus estradiol (79-82%). Under such conditions, HCG caused a very significant shift from estrone to estradiol as was observed in studies with placental minces as well as with homogenates.

Chapter IV

Identification of steroids from studies with human term placenta

These identifications were carried out on extracts in which we wanted to study the percentage conversions of various metabolites from the added 4-^{14}C-labelled precursors. As has been described in methods (page 50-56) at the end of the final incubation period, whenever possible, known tritiated steroids were added to the incubation mixtures. This was done to correct for the losses occuring in the corresponding ^{14}C-products during extraction and purification procedures.

The identification of different steroids will be reported according to their increasing polarity. The steps depicted in methods (page 40-44) for identification are followed here. The final charecterization was carried out on pools of similar reaction products obtained from the various experiments. This was done only after subjecting each individual metabolite to at least two additional chromatographies. Whenever possible, two separate pools of each metabolite were prepared; one from control studies and the other from studies in which HCG was added. Aliquots from these different pools were processed for further purification, formation of derivatives and were finally crystallized to constant specific activity.

A) Androstenedione

This steroid has been detected consistently in all our studies with placenta as one of the conversion product from 4-^{14}C-testosterone. It was initially separated from other steroids in the crude extract in TLC 1. This steroid was then purified in TLC 5, TLC 6 and TLC 3, where it had the chroma--tographic mobility identical to that of authentic androstenedione. After each

chromatography aliquots were taken for counting and constant $^{3}H:^{14}C$ ratios were established (Table XX).

The final charecterization of this compound was carried out by cryst--allizing it to a constant specific activity (269). Table XXI shows four successive crystallizations. In each case $^{3}H:^{14}C$ ratios of crystals and mother liquors are recorded.

B) Estrone

This compound was one of the major radioactive product in placental incubation studies from 4-^{14}C-testosterone as well as from 4-^{14}C-androstenedione. After the elution of this radioactive product from TLC 1, it travelled concurr--ently with authentic estrone standard in TLC 5 and TLC 6. The material was then acetylated with non-radioactive acetic anhydride as described in methods (page 41). The resultant acetate was chromatographed in TLC 6, TLC 2 and TLC 4. Authentic free estrone and its acetate served as standards. After each chroma--tography aliquots were taken to establish $^{3}H:^{14}C$ ratios. The values of these ratios are recorded in Table XXII.

A separate aliquot of estrone monoacetate was also subjected to crystallizations (269). The specific activities of crystals and mother liquors and their $^{3}H:^{14}C$ ratios in each case are shown in Table XXIII.

C) Testosterone

This radioactive metabolite coinciding with the chromatographic behaviour of authentic testosterone was detected in TLC 1, in the studies in which 4-^{14}C-androstenedione was incubated with placental minces. After elution of this steroid from TLC 1, it was rechromatographed in TLC 5, TLC 8 and TLC 3. Acetylation with non-radioactive acetic anhydride was then carried out (page 41) and the testosterone acetate thus formed was chromatographed in

TABLE XX

CHARATERIZATION OF 4-C^{14}-ANDROSTENEDIONE

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE

Chromatographic Systems	$^{3}H:^{14}C$ (c.p.m. ratios)	
	Control	HCG
T L C - 5	0.24	0.21
T L C - 6	0.22	0.20
T L C - 3	0.22	0.20

TABLE XXI

CHARACTERIZATION OF 4-^{14}C-ANDROSTENEDIONE

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

Crystalli--zation Number	Control			HCG		
	^{3}H d.p.m/mg	^{14}C d.p.m/mg	^{3}H:^{14}C ratio(c.p.m)	^{3}H d.p.m/mg	^{14}C d.p.m/mg	^{3}H:^{14}C ratio(c.p.m)
I						
Crystals	7740	10900	0.21	25000	41100	0.18
Mother Liquor	8410	10600	0.23	27200	40700	0.19
II						
Crystals	7640	10800	0.21	25500	41100	0.20
Mother Liquor	7850	10700	0.22	26400	43200	0.21
III						
Crystals	7530	10800	0.21	25600	42000	0.18
Mother Liquor	7600	10700	0.24	25700	41600	0.18
IV						
Crystals	7590	10900	0.22	25600	43600	0.20
Mother Liquor	7620	10800	0.21	25500	42900	0.21

TABLE XXII

CHARACTERIZATION OF 4-^{14}C-ESTRONE

a) ^{3}H:^{14}C RATIOS OF METABOLITE AND DERIVATIVE

Compounds and Chromatographic Systems	^{3}H:^{14}C (c.p.m) ratios	
	Control	HCG
1) ESTRONE (starting material)		
T L C - 1	1.37	1.54
T L C - 5	1.35	1.52
T L C - 6	1.38	1.56
2) ESTRONE MONOACETATE (acetylated product of 1)		
T L C - 6	1.41	1.54
T L C - 2	1.39	1.55
T L C - 4	1.36	1.55

TABLE XXIII

CHARACTERIZATION OF 4-^{14}C-ESTRONE

b) ^{3}H:^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ESTRONE MONOACETATE (ACETYLATED PRODUCT OF ^{14}C-ESTRONE)

Crystalli--zation Number	Control			HCG		
	^{3}H d.p.m/mg	^{14}C d.p.m/mg	^{3}H:^{14}C ratio(c.p.m)	^{3}H d.p.m/mg	^{14}C d.p.m/mg	^{3}H:^{14}C ratio(c.p.m)
I						
Crystals	17800	3790	1.40	26000	4700	1.64
Mother Liquor	17400	3660	1.42	27300	5650	1.44
II						
Crystals	17600	3740	1.40	26200	4700	1.64
Mother Liquor	17800	3780	1.40	26800	4860	1.64
III						
Crystals	18200	3800	1.43	26500	4640	1.66
Mother Liquor	18200	3810	1.40	26400	4720	1.67
IV						
Crystals	18100	3830	1.37	26700	4790	1.66
Mother Liquor	17800	3850	1.37	26900	4780	1.67

TLC 3, TLC 8 and TLC 7. Table XXIV shows these successive steps of identification and $^{3}H:^{14}C$ ratios.

Another aliquot of double labelled testosterone was mixed with 20 mg of authentic testosterone. Four successive crystallizations were carried out from acetone and pentane (269). Table XXV shows the specific activities of crystals and mother liquors and their $^{3}H:^{14}C$ ratios.

D) Estradiol

This radioactive product had been encountered in all the studies with the 4-^{14}C-labelled testosterone and androstenedione. After its initial isolation from TLC 1, the constant $^{3}H:^{14}C$ ratios were obtained in TLC 5 and TLC 6. This compound was acetylated with non-radioactive acetic anhydride (page 41). The resultant diacetate migrated with the same speed as pure estrdiol-17β-diacetate in TLC 6, TLC 2 and TLC 9. (Table XXVI).

A separate aliquot of estradiol was subjected to four successive crystallizations (269). Table XXVII shows the specific activities and $^{3}H:^{14}C$ ratios of crystals and mother liquors.

E) 19-Hydroxy-androstenedione and 19-hydroxy-testosterone

These metabolites appeared in the placental incubation studies when 4-^{14}C-testosterone was used as a precursor. Tritiated authentic 19-hydroxy-androstenedione or 19-hydroxy-testosterone is not commercially available and the amounts of radio-inert materials that we were able to obtain were not more than a few μg. Therefore the identification of these metabolites was limited.

i) 19-Hydroxy-androstenedione:

The radioactive zones having similar mobility to authentic 19-hydroxy-androstenedione in TLC 1 and TLC 5 were pooled from various experiments. The pooled material was purified in TLC 6 and TLC 1. This steroid was then subjected

TABLE XXIV

CHARACTERIZATION OF 4-^{14}C-TESTOSTERONE

a) ^{3}H:^{14}C RATIOS OF METABOLITE AND A DERIVATIVE

Compounds and Chromatographic Systems	^{3}H:^{14}C (c.p.m) ratios	
	Control	HCG
1) TESTOSTERONE (starting material)		
T L C - 5	5.25	3.14
T L C - 8	5.13	3.21
T L C - 3	5.09	3.08
2) TESTOSTERONE ACETATE (acetylation product of 1)		
T L C - 3	4.97	3.17
T L C - 8	5.22	3.14
T L C - 7	5.06	3.26

TABLE XXVI

CHARACTERIZATION OF 4-^{14}C-ESTRADIOL

a) ^{3}H:^{14}C RATIOS OF METABOLITE AND DERIVATIVE

Compounds and Chromatographic Systems	^{3}H:^{14}C (c.p.m) ratios	
	Control	HCG
1) ESTRADIOL (starting material)		
T L C - 1	2.35	2.79
T L C - 5	2.24	2.65
T L C - 6	2.26	2.73
2) ESTRADIOL-17β-DIACETATE (acetylation product of 1)		
T L C - 6	2.29	2.74
T L C - 2	2.33	2.80
T L C - 9	2.41	2.74

TABLE XXVII

CHARACTERIZATION OF 4-^{14}C-ESTRADIOL

b) $^{3}H:^{14}C$ RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

Crystalli-zation Number	Control			HCG		
	^{3}H d.p.m/mg	^{14}C d.p.m/mg	$^{3}H:^{14}C$ ratio(c.p.m)	^{3}H d.p.m/mg	^{14}C d.p.m/mg	$^{3}H:^{14}C$ ratio(c.p.m)
I						
Crystals	20600	2730	2.31	43200	4670	2.75
Mother Liquor	28400	3210	2.82	49200	4420	3.32
II						
Crystals	23500	2770	2.53	42900	4680	2.73
Mother Liquor	26300	3010	2.60	45600	4620	2.95
III						
Crystals	21600	2720	2.37	43100	4700	2.73
Mother Liquor	22500	2830	2.47	43200	4590	2.84
IV						
Crystals	22000	2740	2.39	43500	4770	2.60
Mother Liquor	23100	2790	2.42	43300	4610	2.76

to acetylation with non-radioactive acetic anhydride (page 41). At the same time authentic cold 19-hydroxy-androstenedione (30 μg) was subjected to acetylation with tritiated acetic anhydride as described in methods (page 41-42). The resultant ^{3}H-acetate was purified in TLC 3, TLC 6 and TLC 2. This ^{3}H-19--hydroxy-androstenedione acetate was added to the ^{14}C-19-hydroxy-androstenedione acetate obtained from the experimental pool. The combined double labelled (^{3}H:^{14}C) 19-hydroxy-androstenedione acetate was chromatographed in TLC 6, TLC 3, TLC 7, TLC 2 and TLC 8. After each chromatography, aliquots were taken for counting and ^{3}H:^{14}C ratios were established (Table XXVIII). Constancy of ^{3}H:^{14}C ratios were taken as a criteria of radiochemical purity of the ^{14}C-metabolite under study.

ii) 19-Hydroxy-testosterone

Similar procedure of identification was carried out for this compound as for 19-hydroxy-androstenedione. After its isolation from TLC 1, the pool of ^{14}C-19-hydroxy-testosterone was purified in TLC 5 and TLC 6. This ^{14}C-material was then subjected to cold acetylation (page 41).

^{3}H-19-Hydroxy-testosterone diacetate (obtained the same way as ^{3}H-19-hydroxy-androstenedione acetate) was purified in TLC 3, TLC 6 and TLC 7. This ^{3}H-diacetate was added to ^{14}C-19-hydroxy-testosterone diacetate obtained from the experimental pool. The combined double labelled (^{3}H:^{14}C) 19-hydroxy-testo--sterone diacetate was chromatographed in TLC 6, TLC 3, TLC 7 and TLC 8. After each chromatography ^{3}H:^{14}C ratios were obtained as shown in Table XXIX. Constancy of ^{3}H:^{14}C ratios was taken as a criteria of radiochemical purity of the ^{14}C--metabolite under study.

TABLE XXVIII

CHARATERIZATION OF 4-^{14}C-19-OH-ANDROSTENEDIONE

a) ^{3}H:^{14}C RATIOS OF 19-OH-ANDROSTENEDIONE ACETATE

(ACETYLATION PRODUCT OF 19-OH-ANDROSTENEDIONE)

Chromatographic Systems	^{3}H:^{14}C (c.p.m) ratios of a derivative of 19-OH-ASD
T L C - 6	1.06
T L C - 3	1.27
T L C - 7	1.52
T L C - 2	1.43
T L C - 8	1.53

TABLE XXIX

CHARACTERIZATION OF 4-^{14}C-19-OH-TESTOSTERONE

^{3}H : ^{14}C RATIOS OF 19-OH-TESTOSTERONE DIACETATE (ACETYLATION PRODUCT OF 4-^{14}C-19-OH-TESTOSTERONE)

Chromatographic Systems	^{3}H : ^{14}C (c.p.m.) ratios of a derivative of 19-OH-testo
T L C - 6	1.93
T L C - 3	2.05
T L C -7	2.15
T L C - 8	2.16

Section 2

Studies with human newborn infant's adrenals

Following administration of 3,000 to 10,000 I.U. of HCG to the newborns, Lauritzen et al (186,181) showed that there was a significant increase in the urinary elimination of dehydroisoandrosterone. This increase was even greater in premature infants. Since there is good reason to believe that during the first weeks of life, adrenal morphology and steroid metabolism are very similar to that of the fetus, it has been suggested that HCG may regulate the supply of C-19 precursors in the fetus for placental synthesis of estrogens (180).

In order to investigate the action of HCG on the adrenals of the newborn infants, incubation studies with adrenal minces from human newborns were carried out using ^{14}C-labelled pregnenolone, dehydroisoandrosterone or testosterone as precursors.

The characterization of individual steroids is reported in Section 2, chapter II of this thesis.

Chapter I

Adrenal minces

A) Effect of HCG on the metabolism of 4-^{14}C-pregnenolone by newborn adrenal minces :

Experimental conditions

Adrenal glands from newborn infants were obtained, processed prior to incubation and incubated as previously described in methods (page 46-47).

Each incubation flask contained 400 mg of adrenal minces in a total volume of 6 ml of KRBG. HCG (5000 I.U.) was added to the experimental flask prior to incubation. Incubations were carried out for 2 hours in the presence of

4-^{14}C-pregnenolone (1,683,200 d.p.m., 13.5 nmol). At the completion of the incubation, the media and tissues were processed separately. They were divided into aliquots referred to thereafter as part I and part II; which were processed as described in methods (page 53-56 and Figure 3). Briefly, the aliquots from part I were extracted with methylene chloride and the extracts were applied on TLC 1. The extracts from part II were acetylated with ^{3}H-acetic anhydride (S.A.: 675,00 d.p.m./nmol) and the acetylated products were applied on TLC 2. Pregneno--lone and its acetate were purified to constant ^{14}C: ^{3}H ratios by sequential chromatography on TLC 4, TLC 5 and TLC 6.

After the extraction of part I with methylene chloride, the residual water was reextracted twice with an equal volume of n-butanol in an attempt to study the formation of steroid conjugates, namely sulfates.

Results

The pattern of metabolites and the percentage conversion of ^{14}C--pregnenolone to different metabolites in the media, the tissues and in the sum of media plus tissue are shown in Table XXX.

The only conversion products that could be isolated from controls, and these in small amounts, were androstenedione (3.3%) and 11β-hydroxy-andro--stenedione (2.5%). Analyzing the conversion of steroids in the sum of media plus tissues, it can be seen that HCG induced a marked decrease in the incorpo--ration of radioactivity into androstenedione (0.3%) and 11β-hydroxy-androst--enedione (0.2%) (Table XXX). When the contents of radioactive metabolites and of the unconverted precursor were analyzed separately in the media and the tissues, there was as much, if not more, incorporation of ^{14}C-precursor in the tissues than in the media.

Table XXXI presents the production of pregnenolone in the media, in the tissue and in the sum of media plus tissue; expressed in nanomoles.

TABLE XXX

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-PREGNENOLONE BY NEWBORN ADRENAL MINCES

%-Conversion from ^{14}C-preg--nenolone to:		Media	Tissue	Media + Tissue
*Pregnenolone	Control	33.7	47.5	81.2
	HCG	26.5	58.1	84.6
ASD	Control	1.5	1.8	3.3
	HCG	0.1	0.2	0.3
11β-OH-ASD	Control	1.1	1.4	2.5
	HCG	0.1	0.1	0.2

Each incubation contained 400 mg of adrenal minces in a final volume of 6 ml KRBG. HCG was used in the concentration of 5,000 I.U. per incubation. Incubations were carried out for two hours.

* Unconverted substrate recovered.

TABLE XXXI

EFFECT OF HCG ON THE PRODUCTION OF PREGNENOLONE (in nmol) BY NEWBORN ADRENAL MINCES

	Media			Tissue			Media + Tissue		
	T.P.	Ex. P.	En. P.	T.P.	Ex. P.	En. P.	T.P.	Ex. P.	En. P.
Control	5.0	3.7	1.3	7.6	4.9	2.7	12.6	8.6	4.0
HCG (5000 I.U.)	4.8	3.5	1.3	11.7	6.8	4.9	16.5	10.3	6.2

Each incubation contained 400 mg of adrenal minces in a final volume of 6 ml KRBG. Incubations were carried out for two hours.

T.P. — Total production, Ex. P. — Exogenous production, En. P. — Endogenous production.

The increase in the endogenous production of pregnenolone observed in the tissues (2.7-4.9 nmol), compared to the media (1.3 nmol), is in accordance with the fact that pregnenolone is a precursor and is more bound to the tissues. The endogenous production in the sum of media plus tissue was higher when HCG was present in the incubation mixture (control: 4.0 nmol, HCG:6.2 nmol).

The endogenous production of androstenedione and 11β-hydroxy-andros--tenedione could not be determined due to the small percentage conversions to these steroids.

B) Effect of HCG on the metabolism of 4-^{14}C-dehydroisoandrosterone by newborn adrenal minces:

Experimental conditions

two similar experiments were carried out with 400 mg of newborn infant's adrenal minces. Each incubation consisted of 3 flasks supplemented as follows:

Experiment 1: flask 1. control.
flask 2. HCG (500 I.U.).
flask 3. HCG (3000 I.U.).

Experiment 2: flask 1. control.
flask 2. HCG (3000 I.U.).
flask 3. HCG (6000 I.U.).

The incubations were carried out in the presence of 4-^{14}C-dehydroiso--androsterone (1,802,500 d.p.m., 4.0 μg) in 6 ml of KRBG. In experiment 1, incubations were performed for 1 hour and in experiment 2, for 2 hours.

Purification of dehydroisoandrosterone and its acetate were carried out in the TLC svstems reported for pregnenolone.

Results

The results of experiment 1 and 2 are shown in Table XXXII. They

TABLE XXXII

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-DEHYDROISOANDROSTERONE BY NEWBORN ADRENAL MINCES

Expt. No.		%-Conversion from ^{14}C-DHA to:		
		*DHA	ASD	11β-OH-ASD
1	Control	76.4	7.0	2.1
	HCG (500 I.U)	76.3	5.3	1.4
	HCG (3000 I.U)	78.0	3.1	0.7
2.	Control	57.1	13.8	15.6
	HCG (3000 I.U)	66.4	9.4	6.4
	HCG (6000 I.U)	80.6	1.1	0.9

Each incubation contained 400 mg of adrenal minces in a final volume of 6 ml KRBG. In experiment no. 1, incubations were carried out for one hour and in experiment no. 2, incubations were carried out for two hours.

* Unconverted substrate recovered.

are expressed as percentage conversion of dehydroisoandrosterone to different metabolites.

As obtained in the study with 4-^{14}C-pregnenolone, androstenedione and 11β-hydroxy-androstenedione were the only products detected from 4-^{14}C-dehydro--isoandrosterone.

In experiment 1, addition of 500 I.U. of HCG caused a slight decrease in the formation of androstenedione (5.3%) and 11β-hydroxy-androstenedione (1.4%) compared to control values: 7.0% and 2.1% respectively. Addition of 3000 I.U. of HCG, caused a further decreased in androstenedione (3.1%) and 11β-hydroxy--androstenedione (0.7%). Similar results were obtained in experiment 2; where addition of 6000 I.U. of HCG, caused a very marked decrease in androstenedione and 11β-hydroxy-androstenedione.

C) Effect of HCG on the metabolism of 4-^{14}C-testosterone by newborn adrenal minces in the presence of various co-factores:

In the experiments with placental minces, it was shown that HCG increases the conversion of 4-^{14}C-testosterone to estrone and estradiol, when NADP or NADP plus 3',5'-AMP were present, while it had no effect on the formation of these steroids from 4-^{14}C-testosterone in the presence of G-6-P (Tables V and VI). It was therefore of interest to investigate the action of HCG on the metabolism of 4-^{14}C-testosterone in the newborn infant's adrenals under conditions similar to those employed for placental incubations.

Experimental conditions

Four separate experiments were conducted with newborn infant's adrenals obtained at different times. These are reported as study I and II. Study I, consisted of 2 experiments:

Experiment 1: Incubations were carried out for 1 hour with 400 mg of adrenal

minces in the presence of 4-^{14}C-testosterone (1,925,000 d.p.m., 4.2 μg) in a final volume of 6 ml of KRBG. Each incubation flask was supplemented as follows:

flask 1. control.

flask 2. HCG (500 I.U.).

flask 3. HCG (3000 I.U.).

Experiment 2: Incubations were carried out for 2 hours with 250 mg of adrenal minces in the presence of 4-^{14}C-testosterone (1,089,600 d.p.m., 4.2 μg) in a final volume of 3 ml of KRBG. Each incubation flask was supplemented as follows:

flask 1. control.

flask 2. HCG (3000 I.U.).

flask 3. G-6-P (8.2 μmol).

flask 4. G-6-P (8.2 μmol), HCG (3000 I.U.).

flask 5. NADP (2.6 μmol).

flask 6. NADP (2.6 μmol), HCG (3000 I.U.).

Study II, consisted of 2 experiments:

Experiment 1: Incubations were carried out for 20 minutes with 182 mg of adrenal minces in the presence of 4-^{14}C-testosterone (1,089,600 d.p.m.,4.2 μg) in a final volume of 3 ml of KRBG. Each incubation flask was supplemented as follows:

flask 1. control.

flask 2. NADP (2.6 μmol), G-6-P (8.2 μmol).

flask 3. NADP (2.6 μmol), G-6-P (8.2 μmol), HCG (3000 I.U.).

Experiment 2: Incubations were carried out for 2 hours with 250 mg of adrenal minces in the presence of 4-^{14}C-testosterone (1,089,600 d.p.m., 4.2 μg) in a final volume of 3 ml of KRBG. Each incubation flask was supplemented as follows:

flask 1. control.

flask 2. NADP (2.6 μmol), G-6-P (8.2 μmol).

flask 3. NADP (2.6 μmol), G-6-P (8.2 μmol), HCG (3000 I.U.).

flask 4. NADP (2.6 μmol), G-6-P (8.2 μmol), HCG (6000 I.U.).

Results

The results of study I and II are presented in Tables XXXIII and XXXIV respectively; show the percentage conversion of 4-^{14}C-testosterone to different steroids.

Compounds with the chromatographic behaviour of androstenedione, 11β--hydroxy-androstenedione, 16α-hydroxy-androstenedione, 19-hydroxy-androstene--dione and 19-hydroxy-testosterone were detected as the metabolic products of ^{14}C-testosterone. It must be underlined here that since reference tritiated 11β-hydroxy-androstenedione, 16α-hydroxy-androstenedione, 19-hydroxy-androst--enedione and 19-hydroxy-testosterone are not available, the exact losses of these compounds during the course of extraction and purification procedures could not be precisely established. However, the values were corrected using the mean losses established for other steroids such as; androstenedione and testosterone.

In the control, the percentage conversions were very small, the greater conversion being to 19-hydroxy-testosterone (11.2%) (Table XXXIII). It is only in the presence of 3000 I.U. of HCG that a decrease in the formation of all the metabolites from ^{14}C-testosterone could be observed (Table XXXIII, experiment 1 and 2).

Supplementation of the incubation mixture with G-6-P alone, led to a very slight increase in the percentage yield of androstenedione (1.7%) and 19-hydroxy-androstenedione (2.5%) over control values. Addition of NADP alone, increased more markedly the percentage yield of androstenedione (21%) and also of 11β-hydrosy-androstenedione (0.5%), 16α-hydroxy-androstenedione (3.4%), 19-hydroxy-androstenedione (6%) and 19-hydroxy-testosterone (3.1%). Addition of HCG to the incubation flasks containing either G-6-P or NADP, once more

TABLE XXXIII

EFFECT OF HCG ALONE OR IN THE PRESENCE OF VARIOUS CO-FACTORS ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY NEWBORN ADRENAL MINCES

Expt. No.		%-Conversion from ^{14}C-testosterone to:					
		*TESTO	ASD	11β-OH-ASD	16α-OH-ASD	19-OH-ASD	19-OH-TESTO
1	Control	69.6	1.0	3.1	0.9	-	11.2
	HCG (500 I.U.)	71.3	0.9	2.4	0.7	-	9.9
	HCG (3000 I.U.)	76.0	0.5	1.0	0.5	-	8.1
2	Control	75.7	1.0	-	-	1.0	2.6
	HCG (3000 I.U.)	78.3	0.7	-	-	0.2	0.2
	G-6-P	76.9	1.7	-	0.2	2.5	2.7
	G-6-P + HCG (3000 I.U.)	79.0	1.3	-	-	0.4	0.3
	NADP	52.0	20.9	0.5	3.4	5.9	3.1
	NADP + HCG (3000 I.U.)	77.0	4.8	-	-	0.9	0.5

Expt. No. 1. Each incubation contained 400 mg of adrenal minces in a final volume of 6 ml KRBG. Incubations were carried out for 1 hour.

Expt. No. 2. Each incubation contained 250 mg of adrenal minces in a final volume of 3 ml KRBG. Incubations were carried out for 2 hours.

* Unconverted substrate recovered.

TABLE XXXIV

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-TESTOSTERONE BY NEWBORN ADRENAL MINCES IN THE PRESENCE OF NADPH GENERATING SYSTEM

Expt. No.		*TESTO	%-Conversion from ^{14}C-testosterone to: ASD	11β-OH-ASD	16α-OH-ASD	19-OH-ASD	19-OH-TESTO
1	Control	81.8	0.4	-	-	0.5	1.7
	NADP + G-6-P	70.6	2.7	1.0	2.7	6.1	2.5
	NADP + G-6-P + HCG (6000 I.U.)	77.7	2.2	0.6	0.4	1.8	1.1
2	Control	68.3	3.3	1.7	0.6	2.0	5.1
	NADP + G-6-P	24.2	8.4	8.9	12.0	9.4	16.2
	NADP + G-6-P + HCG (3000 I.U.)	48.5	6.9	6.6	2.1	5.8	13.6
	NADP + G-6-P + HCG (6000 I.U.)	56.8	5.8	2.8	0.8	4.2	12.2

Expt. No. 1. Each incubation contained 182 mg of adrenal minces in a final volume of 3 ml KRBG. Incubations were carried out for 20 minutes.

Expt. No. 2. Each incubation contained 250 mg of adrenal minces in a final volume of 3 ml KRBG. Incubations were carried out for 2 hours.

* Unconverted substrate recovered.

reduced the percentage conversions of these metabolites, compared to their respective controls.

In study II, the combined addition of NADP plus G-6-P to the adrenal minces, increased the production of all the steroids when compared to the values obtained in the controls in the absence of these co-factors (Table XXXIV). The production of steroids in the control and experimental flasks were increased when the incubations were carried out for 2 hours (Table XXXIV,experiment 2). Simultaneous addition of HCG to NADP plus G-6-P, inhibited the action of the NADPH generating system. This inhibition, increased with the dose of HCG.

Discussion

In our studies with newborn adrenal minces, a small conversion of pregnenolone to androstenedione and 11β-hydroxy-androstenedione was demonstrated. This conversion has already been reported in the fetal adrenal minces (122) and in fetal adrenal slices (124). In incubations of newborn adrenal homogenates, Villee and Loring (304) have demonstrated the conversion of pregnenolone to androstenedione, progesterone, 16α-hydroxy-progesterone, cortisol, corticosterone and 17-hydroxy-pregnenolone. We were unable to observe the conversion of pregne--nolone to C-21 steroids.

Our studies show that newborn adrenal minces can convert dehydroiso--androsterone to androstenedione and 11β-hydroxy-androstenedione to a small extent. The conversion of dehydroisoandrosterone to androstenedione has been reported in the homogenates (143) and minces (144) of the fetal adrenals. Incubation studies carried out by Shirley et al (145) have shown the conversion of dehydroisoandrosterone to androstenedione and 11β-hydroxy-androstenedione by the adrenals of an hydrocephalic infant.

We were unable to detect any sulfates of pregnenolone or dehydroiso--androsterone in the butanol extracts of adrenal minces. This may be due to the fact that we did not use calcium chloride ($CaCl_2$) free KRBG buffer as an

incubation medium and that the preincubations were not carried out in the presence of ATP in order to enhance the formation of the active sulfate.

These studies show the presence of 3β-hydroxysteroid dehydrogenase--isomerase in the newborn adrenals. Bloch and Benirschke (116) found low activity of this enzyme, when slices of different zones of the adrenals from a midterm fetus were incubated with acetate. Baillie et al (305) and Niemi and Baillie (306), in a histochemical study, demonstrated the presence of some 3β-hydroxy--steroid dehydrogenase activity in the 'definitive' and 'fetal' cortex of fetuses of various ages, following incubation with pregnenolone, 17α-hydroxy--pregnenolone, dehydroisoandrosterone and androstenediol.

However, the production of large quantities of Δ^5-steroids by the fetus (119) and by the neonate (308) have indicated that 3β-hydroxysteroid dehydrogenase is not very active in fetal tissues. This view is supported by the histochemical studies of Goldman et al (309,310) and Cavallero and Magrini (311), who reported the absence of the enzyme in the 'fetal' zone of the adrenal cortex throughout gestation.

Addition of HCG to the incubation of newborn adrenal minces, produced a decrease in 3β-hydroxysteroid dehydrogenase activity using both pregnenolone and dehydroisoandrosterone as precursors. On the otherhand, HCG increased the endogenous production of pregnenolone in the adrenal (Table XXXI). This increase could be due to the fact that HCG inhibits the enzymatic systems necessary for the formation of different metabolites of pregnenolone; with the result that pregnenolone itself accumulates in the tissue. It could be, on otherhand, that HCG does increase the production rate of pregnenolone by the newborn adrenal.

Solomon et al (10),following the injection of pregnenolone into the umbilical vein at midterm, isolated pregnenolone sulfate, 17α-hydroxy-pregneno--lone sulfate, dehydroisoandrosterone sulfate and dehydroisoandrosterone from the adrenals, but did not detect any Δ^4, 3-ketosteroids. Since in our studies, HCG

inhibited the conversions of Δ^5-steroids to Δ^4-steroids, the deficiency of Δ^5- 3β-hydroxysteroid dehydrogenase reported in the fetus could be due to the presence of HCG in fetal plasma. Indeed HCG has been detected in the human umbilical vein and arteries (180), and in the fetal adrenals (183). The presence of HCG has also been shown in meconium and in the faeces of newborn infants during the first days of life (180-182).

The incubations of newborn adrenal minces with ^{14}C-testosterone, indicated that this precursor could be converted; though to a small extent, to androstenedione, 11β-hydroxy-androstenedione, 16α-hydroxy-androstenedione, 19-hydroxy-androstenedione and 19-hydroxy-testosterone. Following the perfusion of midterm fetuses with androstenedione and testosterone, 11β-hydroxy-androst--enedione was isolated from the adrenals (87). Griffiths (312) was able to demonstrate 19-hydroxylase activity in the human fetal adrenal. It has also been shown that both the fetal adrenal and liver possess the capacity of forming 16α-hydroxylated steroids (117,147,308).

The presence of HCG in the incubations of newborn adrenal minces with ^{14}C-testosterone, led to an overall decrease in the formation of steroids from this precursor, regardless of the co-factors or the length of incubation.

Although definite conclusions are not possible, it is reasonable to believe that HCG acts on the fetal adrenal by inhibition of the Δ^5, 3β-hydroxy--steroid dehydrogenase as well as of other hydroxylase systems (such as 11β-, 16α-and 19-hydroxylases). In placental minces, HCG increased the conversion of testosterone to estrone plus estradiol in the presence of NADP. This would suggest that HCG has specific action on different tissues. It stimulates the aromatizing enzyme systems in the placenta; with the result that there is an increase in the formation of estrogens necessary for the maintenance of pregnancy. On the other hand, in the adrenal gland, it inhibits the conversions of Δ^5 to Δ^4-steroids and the subsequent hydroxylations of Δ^4-steroids . This inhibition

in the adrenal gland would result in increased concentrations of pregnenolone and dehydroisoandrosterone sulfates; in turn these two steroids would be available to the placenta for the synthesis of progesterone and estrogens.

Chapter II

Identification of steroids from studies with human newborn infant's adrenals

The final characterization of different steroids was carried out on pools of similar metabolites, isolated from the various experiments. This was done only after preliminary purification of each steroid in question was established in at least two additional solvent systems. Due to limited number of experiments that we were able to carry out with newborn adrenals, only one pool of each steroid (which represented the studies of controls as well as of HCG) was prepared. The identification of various steroids is described according to their increasing polarity.

A) Androstenedione

This steroid was consistently detected as a conversion product from 4-^{14}C-pregnenolone, 4-^{14}C-dehydroisoandrosterone and 4-^{14}C-testosterone in incubations with newborn adrenal minces. This compound was initially isolated from TLC 1. A pool of androstenedione with ^{3}H (added) and ^{14}C (formed) labels was prepared from various experiments. This ^{3}H:^{14}C pool had an identical chromatographic mobility with the authentic radio-inert androstenedione in TLC 1, TLC 6, TLC 3 and TLC 5 (Table XXXV).

The final characterization of double labelled androstenedione was carried out by crystallizing it to cnstant specific activity (269). Table XXXVI shows the specific activities of crystals and mother liquors and their ^{3}H:^{14}C ratios.

B) 11β-Hydroxy-androstenedione

A ^{14}C-compound with the identical mobility of authentic 11β-hydroxy--androstenedione was detected in TLC 5, in incubation studies with 4-^{14}C-pregne--nolone, 4-^{14}C-dehydroisoandrosterone and 4-^{14}C-testosterone. The tritiated

TABLE XXXV

CHARATERIZATION OF 4-^{14}C-ANDROSTENEDIONE

a) $^{3}H:^{14}C$ RATIOS OF METABOLITE

Chromatographic Systems	$^{3}H:^{14}C$ (c.p.m.) ratios
T L C - 1	0.35
T L C - 6	0.50
T L C - 3	0.49
T L C - 5	0.50

TABLE XXXVI

CHARACTERIZATION OF 4-^{14}C-ANDROSTENEDIONE

b) ^{3}H : ^{14}C RATIOS AND S.A. AFTER SUCCESSIVE CRYSTALLIZATION OF ISOLATED METABOLITE

	^{3}H d.p.m/mg	^{14}C d.p.m/mg	^{3}H : ^{14}C (c.p.m) ratios
I			
Crystals	25600	14400	0.53
Mother Liquor	23900	15200	0.47
II			
Crystals	24600	14900	0.49
Mother Liquor	25600	15000	0.51
III			
Crystals	24800	14800	0.50
Mother Liquor	25300	15200	0.52
IV			
Crystals	24900	14900	0.52
Mother Liquor	25100	15100	0.53

11β-hydroxy-androstenedione is not commercially available and the amounts of ^{3}H-11β-hydroxy-androstenedione that were provided by Dr.A.Z.Mehdi, Notré-Dame Hospital, Montreal, were very limited. After elution of this compound from TLC 5, a pool of ^{14}C-11β-hydroxy-androstenedione was prepared from various experiments. This pool was mixed with known amounts of corresponding ^{3}H-11β-hydroxy-androst--enedione and the carrier (20 μg) 11β-hydroxy-androstenedione. This double labelled (^{3}H:^{14}C) compound was purified in TLC 5, TLC 6 and TLC 1. The material was then subjected to oxidation with chromium trioxide (258). The resultant 11--oxo-compound, i.e., adrenosterone, was chromatographed in TLC 5, TLC 6 and TLC 1. After each chromatography,aliquots were taken for counting and ^{3}H:^{14}C ratios were established (Table XXXVII). Constancy of ^{3}H:^{14}C ratios was taken as a criteria of radiochemical purity of the ^{14}C-metabolite under study.

C) 16α-Hydroxy-androstenedione, 19-hydroxy-androstenedione and 19-hydroxy--testosterone

Zones of radioactivity with identical chromatographic mobility to authentic 16α-hydroxy-androstenedione, 19-hydroxy-androstenedione and 19-hydroxy-testosterone were found in studies with 4-^{14}C-testosterone, using newborn adrenal minces. The tritiated 16α-hydroxy-androstenedione, 19-hydroxy-androstenedione and 19-hydroxy-testosterone are not commercially available. Individual ^{14}C--pools of these three different steroids were prepared from various experiments. Purification of individual pools was carried out in TLC 5, TLC 1, TLC 6 and TLC 3. These individual ^{14}C-pools were then acetylated with non-radioactive acetic anhydride as described in methods (page 41). At the same time, authentic radio-inert 16α-hydroxy-androstenedione (30 μg), 19-hydroxy-androstenedione (30 μg) and 19-hydroxy-testosterone (30 μg) were acetylated with ^{3}H-acetic anhydride (see methods , page 41-42). After the purification of resultant ^{3}H--acetates in various TLC systems, known amounts of ^{3}H-16α-hydroxy-androstene--dione acetate, ^{3}H-19-hydroxy-androstenedione acetate and ^{3}H-19-hydroxy-testo-

TABLE XXXVII

CHARACTERIZATION OF 4-^{14}C-11β-OH-ANDROSTENEDIONE

^{3}H : ^{14}C RATIOS OF METABOLITE AND DERIVATIVE

Compounds and chromatographic systems	^{3}H:^{14}C (cp.m.) ratio
1) 11β-OH-ASD (starting material)	
T L C - 5	0.36
T L C - 6	0.37
T L C - 1	0.37
2) ADRENOSTERONE (oxidized product of 1)	
T L C - 5	0.36
T L C - 6	0.35
T L C - 1	0.35

-sterone diacetate were added to the corresponding ^{14}C-acetates obtained from the experimental incubations. The combined double labelled (^{3}H:^{14}C) acetates of these three steroids were chromatographed in various TLC systems to constant ^{3}H:^{14}C ratios as shown in Table XXXVIII. Constancy of ^{3}H:^{14}C ratios was taken as a criteria of radiochemical purity of the ^{14}C-metabolites under study.

TABLE XXXVIII

CHARACTERIZATION OF 4-^{14}C-16α-OH-ANDROSTENEDIONE, 4-^{14}C-19-OH-ANDROSTENEDIONE AND 4-^{14}C-19-OH-TESTOSTERONE.

^{3}H : ^{14}C RATIOS OF THEIR ACETATE DERIVATIVES

Chromatographic Systems	^{3}H : ^{14}C (c.p.m) ratios of:
	16α-OH-ASD ACETATE
T L C - 6	0.87
T L C - 3	0.96
T L C - 7	0.96
T L C - 8	0.92
	19-OH-ASD ACETATE
T L C - 3	7.02
T L C - 7	7.81
T L C - 2	7.79
T L C - 8	7.87
	19-OH-TESTO DIACETATE
T L C - 6	2.61
T L C - 3	3.29
T L C - 8	3.37
T L C - 7	3.31

Section 3

Studies with rat adrenals

Since there is an obvious limitation of adrenal glands that can be obtained from newborn infats, adrenal glands from rats were used to explore further, the role of HCG on the biosynthesis of certain steroids. Incubation studies were carried out using ^{14}C-labelled pregnenolone, dehydroisoandrosterone, progesterone and deoxycorticosterone.

Chapter I

Adrenal bisects

A) Effect of HCG on the metabolism of 4-^{14}C-pregnenolone by rat adrenal bisects:

Experimental conditions

Adrenal glands from male hooded rats were obtained, processed prior to incubation and incubated as previously described in the methods (page 47).

Each incubation flask contained 228 mg of adrenal bisects in a final volume of 6 ml of KRBG. HCG (3000 I.U.) was added to the experimental flask prior to incubation. Incubations were then allowed to proceed for 2 hours at 37°C under 95% O_2 - 5% CO_2 in the presence of 4-^{14}C-pregnenolone (1,858,700 d.p.m., 4.7 μg).

Since it has been demonstrated that the steroids present in the tissues and in the media do not form a homogenous pool (275), at the completion of the incubation, the media and the tissues were extracted with methylene chlo--ride and processed separately. The aqueous fractions of the media and tissue were divided into part I and part II and were processed as described in detail

by Stachenko et al (275). A scheme of these procedures is presented in Figure 3. To part I of the aqueous fraction, tritiated steroids were added to correct for the experimental losses incurred during purification of ^{14}C-metabolites. From part I, the radioactivity incorporated into different steroids was deter--mined. The specific activity of these different steroids was established in the extract of the aliquot of part II, after acetylation with ^{3}H-acetic anhydride (S.A.: 55,500 d.p.m. / nmol). Knowing the radioactivity incorporated into and the specific activity of each steroid, it was possible to calculate:

a) the production of these different steroids, which is an index of the enzymatic activity of the tissue,

b) the exogenous production, i.e., the part of the total production which is derived from the precursor added , and

c) the endogenous production, which is that part of the total production derived from internal precursors.

Results

The results are presented on Table XXXIX. It should be underlined that except in the instance of the precursor added (i.e., pregnenolone), the exogenous production of individual steroids was always smaller than the endo--genous production. The data show that the values obtained for the total and endogenous production are a better index of the biosynthetic capacity of the tissue.

When the total and endogenous production of the different steroids in the media plus tissues are considered, it can be seen that the quantity of progesterone produced is increased when HCG is present in the media. The production of deoxycorticosterone, on the other hand, is not affected to any great extent by HCG. Progesterone and deoxycorticosterone seem to be bound to the tissue; since their concentrations are higher in the tissue than in the media.

TABLE XXXIX

EFFECT OF HCG ON THE PRODUCTION OF STEROIDS (in nmol) FROM 4-^{14}C-PREGNENOLONE BY RAT ADRENAL BISECTS

Production of steroids in nmol		Media			Tissue			Media + Tissue		
		T.P.	- Ex. P.	= En. P.	T.P.	- Ex. P.	= En. P.	T.P	- Ex. P.	=En. P.
Pregnenolone	Control	1.5	1.4	0.1	1.7	0.4	1.3	3.2	1.8	1.4
	HCG	2.4	2.3	0.1	2.8	2.1	0.7	5.2	4.4	0.8
Progesterone	Control	0.4	0.3	0.1	1.8	0.4	1.4	2.2	0.7	1.5
	HCG	0.4	0.3	0.1	4.3	0.9	3.4	4.7	1.2	3.5
DOC	Control	4.5	1.1	3.4	8.8	0.8	8.0	13.3	1.9	11.4
	HCG	2.6	0.7	1.9	8.6	0.9	7.7	11.2	1.6	9.6
Corticosterone	Control	29.8	1.9	27.9	16.8	0.8	16.0	46.6	2.7	43.9
	HCG	16.7	0.7	16.0	7.0	0.3	6.7	23.7	1.0	22.7
Aldosterone	Control	3.5	0.2	3.3	1.0	0.1	0.9	4.5	0.3	4.2
	HCG	1.0	0.1	0.9	0.4	-	0.3	1.4	0.1	1.3

Each incubation contained 228 mg of rat adrenal bisects in a final volume of 6 ml KRBG. HCG was used in the concentration of 3000 I.U. per incubation. Incubations were carried out for two hours.

T.P. — Total production, Ex. P. — Exogenous production, En. P. — Endogenous production.

However, the production (total or endogenous)of corticosterone and aldosterone are very much decreased in the presence of HCG (Table XXXIX). In contrast to progesterone and deoxycorticosterone, the proportion of corticoster--one and aldosterone released in the media was higher than that bound to the tissue.

The increase in the production of progesterone with HCG is in apparent contradiction with the results obtained with newborn infant's adrenals. Indeed the presence of HCG in the incubation of newborn adrenal tissue induced a marked decrease in the $\Delta^{5},3\beta$-hydroxysteroid dehydrogenase-isomerase activity as manifested by the decreased incorporation of pregnenolone into androstene--dione and 11β-hydroxy-androstenedione (Table XXX). In order to obtain better insight into the activity of the different enzymatic systems of the rat adrenal, the sum of the different steroids possessing 1) a Δ^{4},3-keto group (i.e., pro--gesterone, deoxycorticosterone, corticosterone, aldosterone), 2) a 21-hydroxyl group (i.e., deoxycorticosterone, corticosterone and aldosterone), 3) an 11β--hydroxyl group (i.e., corticosterone and aldosterone) and 4) an 18-hydroxyl group (i.e., aldosterone) were calculated. The values obtained in the media plus tissue are presented on Table XL.

It can be seen that the production of steroids possessing a Δ^{4},3--keto group, a 21-hydroxyl group, an 11β-hydroxyl group and an 18-hydroxyl group were all decreased considerably in the presence of HCG (Table XL) and this was true for the total; the exogenous as well as the endogenous productions.

TABLE XL

EFFECT OF HCG ON THE PRODUCTION OF DIFFERENT STEROIDS (in nmol) FROM 4-^{14}C-PREGNENOLONE BY RAT ADRENAL BISECTS

Productions of steroids (in nmol) from pregnenolone possessing:		Media + Tissue T.P.	- Ex.P.	= En. P.
A Δ^4, 3-Keto group, i.e., Progesterone + DOC + Corticosterone + Aldosterone	Control	66.6	5.6	61.0
	HCG	41.0	3.9	37.1
A 21-Hydroxyl -group, i.e., DOC + Corticosterone + Aldosterone	Control	64.4	4.9	59.5
	HCG	36.3	2.7	33.6
An 11β-Hydroxyl-group, i.e., Corticosterone + Aldosterone	Control	51.1	3.0	48.1
	HCG	25.1	1.1	24.0
An 18-Hdroxyl-group, i.e., Aldosterone	Control	4.5	0.3	4.2
	HCG	1.4	0.1	1.3

Each incubation contained 228 mg of rat adrenal bisects in a final volume of 6 ml KRBG. HCG was used in the concentration of 3000 I.U. per incubation. Incubations were carried out for two hours.

T.P. — Total production, Ex. P. — Exogenous production, En. P. — Endogenous production.

Chapter II

Adrenal subcellular fractions

To investigate further, the inhibiting action of HCG on different enzyme systems, studies were carried out with subcellular fractions of the rat adrenals.

The mitochondrial and microsomal fractions were prepared from fascicu--lata, glomerulosa and whole gland of the rat adrenals as described in methods (page 47-48). It has already to be underlined that what we refer to as "microsomal fraction" is the supernatant of the mitochondrial fraction.

The effect of HCG on 3β-hydroxysteroid dehydrogenase and 21-hydroxy--lase was studied in the microsomal fraction and on 11β-and 18-hydroxylases in the mitochondrial fraction.

A) Assessment of the action of HCG on 3β-hydroxysteroid dehydrogenase-isomerase:

It has been reported that the 3β-hydroxysteroid dehydrogenase--isomerase system is located in the microsomal fraction of adrenal cells. In order to investigate the action of HCG on this enzyme, the microsomal fraction prepared from the fasciculata of rat adrenals was incubated with either 4-^{14}C--pregnenolone or 4-^{14}C-dehydroisoandrosterone. The enzymatic reaction involved in the conversion of pregnenolone to progesterone is mediated by NAD, which acts as a hydrogen acceptor (313). Kowal et al (314,315) using acetone powder prepa--rations of corpus luteum and adrenal cortex, demonstrated that NAD as a co--factor was twice as active as NADP. Either NAD or NADP was added to the incubation media. Two experiments were carried out, one with 4-^{14}C-pregnenolone and the other with 4-^{14}C-dehydroisoandrosterone.

Experimental conditions

Four aliquots of the microsomal fraction of fasciculata equivalent to

0.36 gland of tissue were incubated in the presence of 4-^{14}C-pregnenolone (528,500 d.p.m., 1.3 μg) or 4-^{14}C-dehydrisoandrosterone (448,900 d.p.m., 1.0 μg) for 1 hour at 37^{o}C under 95% O_2 - 5% CO_2. Each incubation flask was supplemented as follows:

flask 1. NAD (3.0 μmol).

flask 2. NAD (3.0 μmol), HCG (2500 I.U.).

flask 3. NADP (2.6 μmol).

flask 4. NADP (2.6 μmol), HCG (2500 I.U.).

The incubates were extracted with methylene chloride and the steroids separated and purified in various TLC systems as reported by Stachenko et al (275).

Results

The percentage conversions of 4-^{14}C-pregnenolone and 4-^{14}C-dehydroiso--androsterone to different steroids are shown in Tables XLI and XLII respectively.

When ^{14}C-pregnenolone was used as a precursor in the presence of NAD or NADP, ^{14}C-labelled progesterone and deoxycorticosterone were isolated. Simultaneous addition of HCG to the incubation mixtures containing NAD or NADP, resulted in a significant decrease in the percentage conversion of these steroids (Table XLI). The decrease induced by HCG was more pronounced in the presence of NADP than in the presence of NAD.

Under the same experimental conditions, when ^{14}C-dehydroisoandrosterone was used as a precursor (Table XLII), the only conversion product isolated was ^{14}C-androstenedione. The presence of HCG, decreased the percentage yield of the latter steroid. This decrease was even more pronounced in the presence of NADP.

B) Assessment of the action of HCG on 21-hydroxylase:

In order to investigate the action of HCG on 21-hydroxylation, aliquots

TABLE XLI

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-PREGNENOLONE BY THE MICROSOMAL FRACTION OF THE FASCICULATA OF THE RAT ADRENAL

Incubation of microsomal fraction with:	%-Conversion from ^{14}C-pregnenolone to:		
	*Pregnenolone	Progesterone	DOC
NAD	17.5	44.7	15.7
NAD + HCG	71.2	11.2	0.1
NADP	6.6	37.6	27.1
NADP + HCG	80.5	0.1	7.8

HCG was used in the concentration of 2500 I.U. per incubation. Incubations were carried out for one hour.

* Unconverted substrate recovered.

TABLE XLII

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-DEHYDROISOANDROSTERONE BY THE MICROSOMAL FRACTION OF THE FASCICULATA OF THE RAT ADRENAL

Incubation of microsomal fraction with:	%-Conversion from ^{14}C-dehydroisoandrosterone to:	
	*DHA	ASD
NAD	4.9	81.6
NAD + HCG	61.6	33.4
NADP	4.5	79.3
NADP + HCG	96.5	0.9

HCG was used in the concentration of 2500 I.U. per incubation. Incubations were carried out for one hour.

* Unconverted substrate recovered.

of microsomal fractions obtained from the fasciculata, glomerulosa or from whole rat adrenal glands were incubated with 4-^{14}C-progesterone.

Experimental conditions

Each fraction was incubated with or without 2500 I.U. of HCG in the presence of 4-^{14}C-progesterone (2,723,700 d.p.m., 8.4 μg), NADP (2.6 μmol), G-6-P (7.2 μmol) and G-6-P-dehydrogenase (5 korngsberg units). The microsomal fraction of the fasciculata was incubated for 15 minutes, that of the whole gland for 30 minutes and that of the glomerulosa for 60 minutes.

After addition of known amounts of tritiated labelled reference steroids, the fractions were extracted with methylene chloride and the extracts processed as in part I (Figure 3) (275).

Results

In Table XLIII are presented the percentage conversions of 4-^{14}C--progesterone to 4-^{14}C-deoxycorticosterone by these microsomal fractions.

It can be seen that the ^{14}C-progesterone which was utilized to a great extent in the controls, particularly by the glomerulosa, was almost not metabolized when HCG was present in the media, so that the percentage conversions of progesterone to deoxycorticosterone in the controls of fasciculata, glomeru--losa and whole glands which were 48.5%, 57.3% and 58.4% respectively were reduced to almost negligible values when HCG was added.

C) Assessment of the action of HCG on 11β-and 18-hydroxylases:

To investigate the action of HCG on the 11β-and 18-hydroxylases, the mitochondrial fraction of the fasciculata, corresponding to the microsomal fraction of the studies reported under (A) and (B), was incubated with 4-^{14}C--deoxycorticosterone.

TABLE XLIII

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-PROGESTERONE BY THE MICROSOMAL FRACTION OF FASCICULATA, GLOMERULOSA AND WHOLE ADRENAL GLAND OF THE RAT

%-Conversion from ^{14}C-progesterone to:		Microsomal fraction from: Fasciculata	Glomerulosa	Whole gland
*Progesterone	Control	27.2	7.2	12.6
	HCG	82.6	79.0	89.4
DOC	Control	48.5	57.3	58.4
	HCG	1.5	1.0	1.0

HCG was used at the concentration of 2500 I.U. per incubation. Microsomal fraction from fasciculata was incubated for fifteen minutes, from glomerulosa for sixty minutes and from whole adrenal gland for thirty minutes. Each incubation flask contained NADP (2.6 μmol), G-6-P (7.2 μmol) and G-6-P-dehydrogenase (5 korngsberg units).

* Unconverted substrate recovered.

Experimental conditions

The separation of the different subcellular fractions of the rat adrenals has been described in methods (page 47-48). Aliquots of the mitocho--ndrial fraction, each corresponding to 3 glands were incubated with 4-^{14}C--deoxycorticosterone (183,500 d.p.m., 0.5 μg) for 30 minutes at 37^{o}C under 95% O_2 - 5% CO_2. Each incubation flask was supplemented with NADP (2.6 μmol), G-6-P (7.2 μmol) and G-6-P-dehydrogenase (5 korngsberg units). HCG (2500 I.U.) was added to the experimental flasks prior to incubation.

At the end of the incubation period, known amounts of ^{3}H-labelled deoxycorticosterone and corticosterone were added to each incubate. The incubates were extracted with methylene chloride. Deoxycorticosterone and corticosterone were separated and purified to constant ^{3}H:^{14}C ratios (275) and the percentage conversions of deoxycorticosterone were calculated. Since ^{3}H-18-hydroxy-11--deoxycorticosterone was not available,the percentage conversion of deoxycorti--costerone to this compound was corrected for experimental losses,from the mean losses of deoxycorticosterone and corticosterone. The identification of 18-hydroxy-11-deoxycorticosterone was carried out by periodic acid oxidation (316).

Table XLIV shows the percentage conversion of 4-^{14}C-deoxycorticosterone to corticosterone and 18-hydroxy-11-deoxycorticosterone.

It can be seen that when HCG was present in the incubation flask, deoxycorticosterone was almost not metabolized. Moreover, the percentage conver--sion to corticosterone and 18-hydroxy-11-deoxycorticosterone, which were 73.4% and 15.3% respectively for the controls, were reduced to negligible values in the presence of HCG.

TABLE XLIV

EFFECT OF HCG ON THE METABOLISM OF 4-^{14}C-DEOXYCORTICOSTERONE BY THE MITOCHONDRIAL FRACTION OF THE FASCICULATA OF THE RAT ADRENAL

%-Conversion from ^{14}C-deoxycorticosterone to:		
*DOC	Control	10.1
	HCG	90.0
Corticosterone	Control	73.4
	HCG	2.9
18-OH-DOC	Control	15.3
	HCG	-

HCG was used in the concentration of 2500 I.U. per incubation. Incubations were carried out for thirty minutes.
Each incubation flask contained NADP (2.6 μmol), G-6-P (7.2 μmol) and G-6-P-dehydrogenase (5 korngsberg units).

* Unconverted substrate recovered.

Discussion

The production of pregnenolone, progesterone, deoxycorticosterone, corticosterone, aldosterone and 18-hydroxy-11-deoxycorticosterone by the rat adrenal as already established by several investigators (261,275,317), has been reconfirmed in the present study. The effect of HCG was to inhibit the production of these steroids from exogenous as well as from endogenous precursors. This implies an inhibition by HCG of $\Delta^5,3\beta$-hydroxysteroid dehydrogenase-isomerase as well as of 21-,11β- and 18-hydroxylases. This inhibition could be demonstra--ted at at the mitochondrial and microsomal levels in cells originating from different zones of the rat adrenal cortex. It must be underlined however, that in incubations of the "microsomal fraction", previously defined as the super--natant of the mitochondrial pellet, NAD or NADP were the only co-factors added. Under such conditions, the 21-hydroxylase activity was efficient. This indicates that addition of G-6-P and G-6-P-dehydrogenase is not absolutely necessary and that these two factors, as well as others, are available in the supernatant for generation of NADPH.

The inhibition in rat adrenal preparations of different enzymatic systems by HCG has been very consistent in all our experiments. Studies with newborn infant's adrenals also showed a decrease in the formation of steroids from pregnenolone, dehydroisoandrosterone and testosterone in the presence of HCG (see Section 2).

However, HCG causes hypertrophy of interstitial cells of the testis (318) and has been shown to increase the incorporation of ^{14}C-acetate into testosterone by testis slices from various species (319). Laatikainen et al (320) have shown that dehydroisoandrosterone, testosterone and androstenedione concentrations in the spermatic vein of the human testis were increased following the injection of HCG. Samuels et al (321), following the injection of HCG to rats, observed a decrease in 3β-hydroxysteroid dehydrogenase activity in the

rat adrenal, while this activity increased rapidly in the testis. It has also been reported (307) that HCG will stimulate ovarian but not adrenal androgen production. However, Pauerstein and Solomon (256) have demonstrated that administration of 20,000 I.U. of HCG per day to three ovariectomized women (aged 24-30 years), caused a definite rise in urinary excretion of etiocholanolone and dehydroisoandrosterone, but androsterone excretion was not affected. The authors concluded that HCG has a qualitative as well as a quantitative effect on adrenal steroid biosynthesis and on the subsequent peripheral metabolism of steroids.

These observations, together with our own, suggest that the action of HCG is heterogenous. HCG has different effects on various enzymes, depending upon the target organ involved. In the rat adrenal, the inhibitory action of HCG on $\Delta^{5},3\beta$-hydroxysteroid dehydrogenase, 21-,11β- and 18-hydroxylases at the subcellular level suggests that this hormone acts directly on these enzyme systems.

It is of interest to underline that in incubations of the microsomal fraction, the inhibition of the conversion of pregnenolone or dehydroisoandro--sterone to progesterone or androstenedione respectively was significantly smaller in the presence of NADP plus HCG than of NAD plus HCG. It is thus possible, that HCG does not act directly on enzymes but that it increases the availability of some co-factors, which are necessary for the activity of such enzymes.

VI. GENERAL DISCUSSION

For each individual study, a discussion of the results has been given. In this section, the significance and implications of the different findings will be discussed mainly in relation to the action of HCG in placental and adrenal tissues.

These studies leave little doubt that human placental minces, homogenates and subcellular fractions can convert C-19 steroids to estrogens. The incubations of such placental preparations with ^{14}C-androstenedione or ^{14}C-testosterone have substantiated previous observations, i.e., isolation of androstenedione (74-76, 283), estrone, estradiol (86,91,293-300) and 19-hydroxy--androstenedione (83,293,294,297,322). When placental homogenates or subcellular fractions were incubated with ^{14}C-testosterone, 19-hydroxy-testosterone was also detected.

The conversion of androstenedione to estrone and estradiol involves the removal of the C_{19} angular methyl group and the introduction of a double bond in ring A. The isolation of steroids hydroxylated at C_{19}, such as, 19--hydroxy-androstenedione and 19-hydroxy-testosterone would suggest that they might be intermediary products in the conversion of androstenedione and testosterone to estrogens. Meyer (83) suggested that 19-hydroxy-androstenedione is an intermediate in the conversion of androstenedione to estrone and estradiol. Longchampt et al (293) incubated ^{14}C-androstenedione with human placental microsomes and obtained ^{14}C-19-hydroxy-androstenedione. The latter compound gave rise to ^{14}C-estrone, when incubated with human placental microsomes. Hayano et al (322) and Morato et al (294) have shown that 19-hydroxy-androstene--dione is converted to estrogens much more rapidlythan androstenedione. Although the participation of 19-hydroxy-androstenedione as an intermediate has been challenged by Hollander (323), kinetic experiments conducted by Wilcox and

Engel (297) provide evidence that 19-hydroxy-androstenedione is an obligatory intermediate between androstenedione and estrogens and suggest that 19-hydroxy--lation is the rate limiting step in aromatization.

It is now generally recgnized that NADPH is the required nucleotide mediating steroid hydroxylations and aromatization. From the incubations of placental preparations in the presence of various co-factors, it was observed that, 1) the addition of NADP to placental minces or to homogenates increased the activity of enzymes associated with aromatization, 2) the addition of G-6-P only, did not affect the activity of aromatizing enzymes and 3) addition of NADP plus G-6-P to the minces, homogenates, or subcellular fractions, activated the aromatizing systems to the extent that an almost complete transformation of testosterone to estrone and estradiol took place. These results indicate that the placental preaparations generated NADPH from the NADP added. It can be concluded that NADP is not sufficient or is not readily available in our placental preparations, whereas, G-6-P is relatively more readily available.

In placental minces, the increase in aromatizing activity induced by NADP was further enhanced by the addition of HCG and/or cyclic 3′,5′-AMP. These results are interpreted as indicating that HCG and cyclic 3′,5′-AMP increase the intracellular concentration of G-6-P. In support of this view, Sutherland (324) has proposed that tropic hormones activate the formation of cyclic 3′,5′-AMP. Haynes et al (285) have shown that ACTH acts in the adrenal gland by initially increasing the intracellular concentration of cyclic 3′,5′-AMP, which in turn increases G-6-P concentration in the gland. The latter when metabolized via the pentose pathway, produces increased amounts of NADPH. Lefkowitz et al (325) and Schimmer et al (326) have indicated that ACTH, after binding to the plasma membrane of the adrenal cell, activates adenyl cyclase on the cellular membrane, which promotes the formation of cyclic 3′,5′-AMP in the cell. Recent studies by Garren et al (327) have demonstrated that the

administration of ACTH to animals, results in a stimulation of DNA synthesis in the adrenal; concomitantly, there occurs an activation of DNA polymerase and thymidine kinase. It remains to be seen whether HCG acts in a similar manner in the placenta.

The stimulation of C-19 steroid aromatization by HCG is in accord--ance with the findings of Cedard et al (109-111). These authors showed a significant increase in the conversion of various C-19 steroids to estrogens, following perfusion of term placentas with HCG.

From the results of different studies in placental minces versus homogenates or subcellular fractions, it is concluded that the stimulatory action of HCG on C-19 steroid aromatization is possible only in intact cells. Indeed an increase in the conversion of testosterone to estrone and estradiol in the presence of HCG (and NADP) was observed only in incubations of placental minces and not in homogenates or subcellular fractions.

The above conclusion can be compared with that of Hechter et al (301), who stated that to demonstrate the action of ACTH on steroidogenesis, intact cells are required. This has been subsequently confirmed by other authors too (288, 289).

However, there are several differences between the 'in vitro' action of ACTH and of HCG. Without supplementation of NADP to the placental minces, HCG inhibited the formation of estrogens. This could imply either 1) that NADP is not readily available in our placental preparation as it is in the adrenal tissue, or 2) that the absence of NADP in our placental preparation is an artefact of the experimental procedure. Cedard et al (111) have reported in perfusion studies of term placenta that addition of G-6-P to the perfusion fluid, increased estrogen production from testosterone as markedly as HCG. Another difference is that, the stimulation of corticosteroid synthesis by ACTH takes place between cholesterol and pregnenolone; thereby increasing the concentrations of

pregnenolone in the gland, whereas HCG stimulates the aromatization of C-19 steroids. In 'in vitro' studies, it has been reported that HCG does not influence the biosynthesis of progesterone by the placenta (328). Administration of high doses of HCG to pregnant women at term, does not in fact alter plasma progesterone levels (277). Nevertheless, more studies are needed to establish whether HCG has any action on the formation of pregnenolone or progesterone by the placenta.

It is interesting that there was always a higher production of 17β--hydroxy-steroids, when HCG was added to the incubations of placental minces, homogenates and in certain instances, subcellular fractions. This was mainly evident from higher concentrations of estradiol than of estrone. These findings indicate that another action of HCG in the placenta could be to protect the 17β -hydroxyl group. Toren and Gordon (112) have suggested that HCG might affect estrogen interconversions by acting as a co-factor in enzymatic reactions. An alternative may be that, HCG preferentially stimulates placental enzymes, which promote the conversion of testosterone to 19-hydroxy-testosterone and estradiol rather than to estrone, estradiol being biologically a more potent estrogen. A similar type of selective alteration of the pathways of steroid metabolism has been demonstrated for thyroid hormone in the instance of androgen metabolites. Gallagher (329) has shown that reduced thyroid function will alter the ratio of androsterone/etiocholanolone in favour of etiocholanolone, while increased thyroid activity changes this ratio in favour of androsterone.

The effect of HCG on the aromatization of testosterone by placental minces was hindered for one to two hours. This delayed effect could be due to a long sequence of intracellualr enzymatic reactions. When HCG was administered to women to promote follicle maturation and ovulation, a delay in its action has been reported. Geist (330) observed luteinization of the theca cells 36 to 100 hours, after a single injection of HCG. Utilizing larger and repeated doses

BIBILIOGRAPHY

1. DICZFALUSY, E., PION, R. and SCHWERS, J. Arch. Anat. microsc. Morphol. Exp. (Paris) 54: 67, 1965.

2. VILLEE, C.A. American J. of Obste. and Gynec. Vol. 104: No 3, 406, 1969.

3. SOLOMON, S.J. Clin. Endocr. 26: 762, 1966.

4. DICZFALUSY, E. Fed. Proc. 23 : 791, 1964.

5. SITTERI, P.K. and MacDONALD, P.C. J. Clin. Endocr. 26 : 751, 1966.

6. DICZFALUSY, E. and BENAGIANO, G. In: Research on Steroids, Vol. 2, p. 27, Editor: C.Cassano. II Pensiero Scientifico, Rome, 1966.

7. DICZFALUSY, E. In: Proceedings, II International congress on hormonal Steroids, Milan, 1966, p. 82-95, Editors: L.Martini, F.Fraschini, and M.Motta. ICS 132, Excerpta Medica, Amsterdam.

8. LEVITZ, M., DANCIS, J., GOEBELSMANN, U., and DICZFALUSY, E. In: Proceedings, II International Congress on Hormonal Steroids, Milan, 1966, p. 646-652. Editors: L.Martini, F.Fraschini, and M.Motta. ICS 132, Excerpta Medica, Amsterdam.

9. MITCHELL, F.L. Vitam. Horm. 25, 191, 1967.

10. SOLOMON, S., BIRD, C.E., LING, W., IWAMIYA, M. and YOUNG, P.C.M. Recent Progr. Hormone Res. 32, 297, 1967.

11. SOLOMON, S. and FRIESEN, H.G. Ann. Rev. Med. 19, 399, 1968.

12. DICZFALUSY, E. In: The Feto-placental Unit, Proceedings of an Internatio--nal Symposium, Milan, 1968, Excerpta Medica, Amsterdam, p. 6509. Editors: A.Pecile and C.Finzi. ICS 183.

13. YOUNGLAI, E.V., STERN, M., LING, W., LEUNG, K. and SOLOMON, S. an International Symposium's Proceedings, In:The Feto-placental unit, Mlian 1968, p.190-200. Editors: A.Pecile and C.Finzi. ICS 183, Excerpta Medica, Amsterdam.

14. SOLOMON, S. and YOUNGLAI, E.V. In: Fetus and placenta, p.249-298. Editors: Arnold Klopper and Egon Diczfalusy. Blackwell Scientific Publications, Oxford and Edinburgh, 1969.

15. DICZFALUSY, E. and MANCUSO, S. In: Fetus and placenta, p. 191-248. Editors: Arnold Klopper and Egon Diczfalusy. Blackwell Scientific Publications, Oxford and Edinburgh, 1969.

16. SOLOMON, S. and FUCHS, F. In: Endocrinology of Pregnancy, p. 66-100. Editors: Fritz Fuchs and Arnold Klopper, Medical Dept.,Harper and Row Publishers, New York, Evanston and London, 1971.

17. HALBAN, J. Geburtshilfe Z Gynaekol, 53: 191, 1904.

18. HALBAN, J. Arch Gynaekol., 75: 353, 1905.

19. ALLEN, E. Am. J. Anat. 30: 297, 1922.

20. MAZER, C. and GOLDSTEIN, L. "Clinical Endocrinology of the Female", p.350, Saunders, Phila., Pa., 1932.

21. DICZFALUSY, E. Acta Endocrinol. 10: 373, 1952.

22. PEARLMAN, W.H. and CERCEO, E.J. J. Biol. Chem., 189: 79, 1952.

23. SALHANICK, H.A., NOALL, M.W., ZARROW, M.X., and SAMUELS, L.T. Science 115: 708, 1952.

24. PHILIPP, E. Zentr. Gynaekol 54: 450, 1930.

25. JONES, G.E.S., GEY, G.O. and GEY, M.K. Bull. Johns Hopkins Hosp. 72: 26, 1943.

26. VILLEE, D.B. New England J. of Medicine, 281: 473, 1969.

27. STEINBERGER, E. and PERLOFF, W.H., Am. J. M. Sc. 247: 503, 1964.

28. NEUWEILER, W. and HERRMANN, Gynaecologia 135: 219, 1953.

29. WEBER, J. Acta Obst. et Gynec. Scandinav, 40: 139, 1961.

30. THIEDE, H.A. and CHOATE, J.W. Obst. and Gynec. 22: 433, 1963.

31. THOMAS, J.B. "Introduction to human Embryology", p. 260 by Lea and Febiger, 1968.

32. VAN LEUSDEN, H. and VILLEE, C.A., Steroids 6: 31, 1965.

33. ZELEWSKI, L. and VILLEE, C.A. Bio Chemistry 5: 1805, 1966.

34. LEVITZ, M., EMERMAN, S. and DANCIS, J. In: Abstracts, International Congress on Hormonal Steroids, Milan, 1962, p. 266. ICS 51, Excerpta Medica, Amsterdam.

35. TELEGDY, G., WEEKS, J.W., LERNER, U., SATKEMANN, G. and DICZFALUSY, E. Acta Endo. 63:91,1970.

36. TELEGDY, G., WEEKS, J.W., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. 63: 105, 1970.

37. TELEGDY, G., WEEKS, J.W., ARCHER, D.F., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. 63: 119, 1970.

38. MATHUR, R.S., ARCHER, D.F., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. 65: 663, 1970.

39. SOLOMON, S., LENZ, A.L., VANDE WIELE, R. and LIEBERMAN, S. In: Abstracts, 126 th. Meeting of the American Chemical Society, New York, p.29C, 1954.

40. JAFFE, R.B., ERIKSSON, G. and DICZFALUSY, E. In: Abstracts, VI Pan American Congress of Endocrinology, Mexico, D.F. 1965. ICS 99, Excerpta Medica, Amsterdam.

41. JAFFE, R.B. and PETERSON, E.P. Steroids,8: 695,1966.

42. SOLOMON, S. "The placenta and fetal membranes", p.201, 1960, Editor: C.A.Villee, Williams & Wilkins Co., Baltimore, Md.

43. MAEYAMA, M., TUCHIDA, Y. and MATUOKA, H. Steroids,11: 799, 1968.

44. MORRISON, G., MEIGS, R.A. and RYAN, K.J. Steroids,6: Suppl. II, 117, 1965.

45. PEARLMAN, W.H., CERCEO, E., and THOMAS, M.J. Biol. Chem. 208: 231, 1954.

46. SOBREVILLA, L., HAGERMAN, D. and VILLEE, C. Biochim. Biophys. Acta 93: 665, 1964.

47. PION, R., JAFFE, R., ERIKSSON, G., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. 48: 234, 1965.

48. JAFFEE, R.B. and LEDGER, W.J. Steroids,8: 61, 1966.

49. PALMER, R., ERIKSSON, G., WIQVIST, N. and Diczfalusy, E. Acta Endocr. 52: 598, 1966.

50. PION, R., CONRAD, S.H. and WOLF, B.J. J. Clin. Endocr. 26: 225, 1966.

51. JUNGMANN, R.A. and SCHWEPPE, J.S. J. Clin. Endocr. 27: 1151, 1967.

52. PALMER, R., BLAIR, J.A., ERIKSSON, G. and DICZFALUSY, E. Acta Endocr. 53: 407, 1966.

53. KITCHIN, J.D., PION, R.J. and CONRAD, S.H. Steroids,9: 263, 1967.

54. JUNGMANN, R.A., KOT, E. and SCHWEPPE, J.S. Steroids, 8: 977, 1966.

55. RYAN, K.J., MEIGS, R. and PETRO, Z. Am. J. Obst. et Gynec., 96: 676, 1966.

56. KITCHIN, J.D., OUYANG, P., CONRAD, S.H. and PION, R.J. Am. J. Obst. et Gynec. 103: 48, 1969.

57. WARREN, J.C. and CHEATUM, S.G. Canad. J. Biochem. 42: 143, 1964.

58. LITTLE, B., SHAW, A. and PURDY, R. Acta Endocr. 43: 510, 1963.

59. JAFFE, R.B., PION, R., ERIKSSON, G., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. (Kbh) 48: 413, 1965.

60. LITTLE, B., DIMARTINIS, J. and NYHOLM, B. Acta Endocr. (Kbh), 30: 530, 1959.

61. DE MIQUEL, M. and TOREN, P. In: Abstracts II, International Congress on Hormonal Steroids, Milan, 1966. p.292, Editors: E.B.Romanoff and L.Martini, ICS III, Excerpta Medica, Amsterdam.

62. ZANDER, J. In: Proceedings, II International Congress of Endocrinology, London, 1964. p.715. Editor: S.Taylor, ICS 83, Excerpta Medica, Amsterdam.

63. HART, D.MCK. J. Endocr. 35: 255, 1966.

64. OSINSKY, P.A., Nature (Lond.), 187: 777, 1960.

65. BERLINER, D.L., JONES, J.E. and SALHANICK, H.A., J.Biol. Chem. 223: 1043, 1956.

66. EASTERLING, Jr., W.E., SIMMER, H., DIGNAM, W.J., FRANKLAND, M.W. and NAFTOLIN, F. Steroids, 8: 157, 1966.

67. SIMMER, H.H., DIGNAM, W.J., EASTERLING, Jr., W.E., FRANKLAND, M.V. and NAFTOLIN,F. Steroids, 8: 179, 1966.

68. BAULIEU, E., WALLACE, E. and LIEBERMAN, S. J. Biol. Chem. 238: 1316, 1963.

69. BLOCH, E. and NEWMAN, E. Endocrinology, 79: 524, 1966.

70. DELL' ACQUA, S., MANCUSO, S., ERIKSSON, G., RUSE, J.L., SOLOMON, S. and DICZFALUSY, E. Acta Endocr. (kbh), 55: 401, 1967.

71. REYNOLDS, J.W., MANCUSO, S., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. (Kbh), 58: 377, 1968.

72. GOVAERTS, M., VAN CROMBREUCQ, T. and SCHWERS, J. In: Abstracts, III Inter--national Congress of Endocrinology, Mexico, D.F., 1968, p. 127. Editor: C.Gual. ICS 157, Excerpta Medica, Amsterdam.

73. STARKA, L., SULCOVA, J. and JIRASEK, J.E. In: Abstracts, International Symposium on feto-placental unit, Milan, 1968, p. 33. Editors: A.Pecile and G.B.Caruthers, ICS 170, Excerpta Medica, Amsterdam.

74. LAMB, E., MANCUSO, S., DELL' ACQUA, S., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. (kbh), 55: 263, 1967.

75. BOLTE, E., MANCUSO, S., DRAY, F., BAULIEU, E.E. and DICZFALUSY, E. Steroids, 4: 613, 1964.

76. BENAGIANO, G., KINCL, F.A., ZIELSKE, F., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. (Kbh), 56: 203, 1967.

77. FRENCH, A.P. and WARREN, J.C. Steroids, 8: 79, 1966.

78. BENAGIANO, G., MANCUSO, S. and DICZFALUSY, E. In: Research on Steroids, Vol. 3, p. 123, 1968. Editor: C.Cassano, II Pensiero Scientifico, Rome.

79. PULKKINEN, M.O. Acta Physiol., Scand., 52: Suppl. 180, 1, 1961.

80. WARREN, J.C. and TIMBERLAKE, C.E. J.Clin. Endocr., 22: 1148, 1962.

81. BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. (Kbh), 45: 535, 1964.

82. BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. (Kbh), 45: 560, 1964.

83. MAYER, A.S. Biochim. Biophys. Acta, 17: 441, 1955.

84. RYAN, K.J. Fed. Proc., 17: 138, 1958.

85. RYAN, K.J. Biochim. Biophys. Acta, 27: 658, 1958.

86. RYAN, K.J. J. Biol. Chem. 234: 268, 1959.

87. MANCUSO, S., BENAGIANO, G., DELL'ACQUA, S., SHAPIRO, M., WIQVIST, N. and DICZFALUSY, E. Acta Endocr. (Kbh), 57: 208, 1968.

88. MANCUSO, S., DELL'ACQUA, S., ERIKSSON, G., WIQVIST, N. and DICZFALUSY, E. Steroids, 5: 183, 1965.

89. MORATO, T., LEMUS, A.E. and GUAL, C. Steroids, 5: Suppl. 1, 59, 1965,

90. WU, C., TOUCHSTONE, J.C. and FLICKINGER, G.L. Am.J. Obst. et Gynec. 102: 862, 1968.

91. CHARREAU, E., JUNG, W., LORING, J. and VILLEE, C. Steroids, 12: 29, 1968.

92. BAGGET, B., ENGEL, L.L., BALDERAS, L., LANMAN, G., SAVARD, K. and DORFMAN, R.I. Endocr. 64: 600, 1959.

93. JACKANICZ, T.M. and DICZFALUSY, E. Steroids, 11: 877, 1968.

94. DELL'ACQUA, S., MANCUSO, S., ERIKSSON, G. and DICZFALUSY, E. Biochim. Biophys. Acta 130: 241, 1966.

95. RYAN, K.J., J. Biol. Chem. 234: 2006, 1959.

96. RYAN, K.J. Endocr. 63: 392, 1958.

97. RYAN, K.J. and ENGEL, L.L., Endocrinology, 52: 287, 1958.

98. LEVITZ, M., CONDON, G.P. and DANCIS, J., Endocrinology, 58: 376, 1956.

99. CANFIELD, R.E. and BELL, J.J., Progress in Endocrinology, proceedings of the third International Congress of Endocrinology, Mexico, D.F. 1968. p. 402, Editor: Gual, C. and Ebling, F.J.G.

100. VENNING, E.H., Textbook of Gynecologic Endocrinology, Editor: J.J.Gold, Holber Medical Division, New York, 1968.

101. DICZFALUSY, E., Acta Endocrinol.(Kbh), Suppl. 12, 1953.

102. DICZFALUSY, E. and TOREN, P., Vitamins and Hormones, 19: 229, 1961.

103. SIMMER, H.H., Biology of Gestation, Vol. 1, Editor: N.S.Assali,Academic Press, New York, 1968.

104. MIKHAIL, G. and ALLEN, W.M., Progr. 46th. Meeting, Endocrine Soc., San Francisco, Calif., p. 37, 1964.

105. RYAN, K.J., Acta Endocrinol., 44: 81, 1963.

106. HAMMERSTEIN, J., RICE, B.F. and SAVARD, K.J., Clin. Endocrinol. Metab., 24: 597, 1964.

107. DEANESLY, R., In: "Marshall's Physiology of Reproduction"(A.S.Parker,ed.) Vol. III, p. 891, 1966. Longmans, Green, New York.

108. LAURITZEN, C., Gynaekol. Rundschau, 3: 1, 1966.

109. CEDARD, L., VARANGOT, J. and YANNOTTI, S.C.R., Acad. Sci. (Paris), 258: 3769, 1964.

110. CEDARD, L., VARANGOT, J. and YANNOTTI, S.C.R., Acad. Sci. (Paris), 262: 379, 1966.

111. CEDARD, L., ALSAT, E., URTASUN, M.J. and VARANGOT, J., In: The feto--placental Unit,Proceedings of an International Symposium, Milan, 1968, p. 207, Editors: A.Pecile and C.Finzi. ICS 183, Excerpta Medica, Amsterdam.

112. TOREN, P. and GORDON, E.E., J. of Clin. Invest., 37: 1516, 1958.

113. LANMAN, J.T., Medicine, (Baltimore), 32: 389, 1953.

114. BLOCH, E. and BENIRSCHKE, K., J. Biol. Chem. 234: 1085, 1959.

115. BLOCH, E., and BENIRSCHKE, K., Acta Endocr. (kbh), Suppl. 51: 691, 1960.

116. BLOCH, E. and BENIRSCHKE, K., In: The Human Adrenal Cortex, 1962, p. 589, Editors: A.R.Currie, T.Symington, and J.K.Grant. E.&S.Livingstone Ltd., Edinburgh.

117. VILLEE, D.B., ENGEL, L.L., LORING, J.M. and VILLEE, C.A., Endocrinology, 69: 354, 1961.

118. RICE, B.F., JOHANSON, C.A. and STERNBERG, W.H., Steroids, 7: 79, 1966.

119. EBERLEIN, W.R., J. Clin. Endocr. 25: 1101, 1965.

120. CONRAD, S.H., PION, R.G. and KITCHIN III, J.D., J.Clin. Endocr. 27: 114, 1967.

121. VILLEE, C.A., LORING, J.N. and VILLEE, D.B., In: Abstracts, International Congress in Hormonal Steroids, Milan, 1962, p. 143. ICS 51, Excerpta Medica, Amsterdam.

122. VILLEE, D.B. and DRISCOLL, S.G., Endocrinology, 77: 602, 1965.

123. SHAHWAN, M.M., OAKEY, R.E. and STITCH, S.R., In: Abstracts, III Inter--national Congress of Endocrinology, Mexico, D.F., 1968. p. 172. Editor: C.Gual, ICS 157, EXCERPTA Medica, Amsterdam.

124. MILNER, A.J. and MILLS, I.H., J.Endocr. 47: 369, 1970.

125. JACKANICZ, T.N., WIQVIST, N. and DICZFALUSY, E., Biochim. Biophys, Acta. 176: 883, 1969.

126. REYNOLDS, J.W., WIQVIST, N. and DICZFALUSY, E., Acta. Endocr. 61: 533, 1969.

127. MAEYAMA, M., MATUOKA, H., TUCHIDA, Y. and HASHIMOTO, Y., Steroids, 15:167, 1970.

128. PION, R.J., JAFFE, R.B., WIQVIST, N. and DICZFALUSY, E., Biochim. Biophys. Acta. 137: 584, 1967.

129. PEREZ-PALACIOS, G., PEREZ, A.E. and JAFFE, R.B., J. Clin. Endocr. 28: 19, 1968.

130. HARBERT, Jr., G.M., Mc GAUGHEY, Jr., H.S., SCOGGIN, W.A. and THORTON, W.M, Obst. et Gynec., (N.Y.), 23: 413, 1964.

131. BENGTSSON, G., ULLBERG, S., WIQVIST, N. and DICZFALUSY, E., Acta Endocr. (Kbh), 46: 544, 1964.

132. SOLOMON, S., LANMAN, J.T., LIND, J. and LIEBERMAN, S.,J. Biol. Chem. 233: 1084, 1958.

133. VILLEE, D.B., ENGEL, L.L. and VILLEE, C.A., Endocrinology, 65: 465, 1959.

134. BLOCH, E., ROMNEY, S.L., KLEIN, M., LIPPIELLO, L., COOPER, P. and GOLDRING, I.P., Proc. Soc. Exp.Biol. Med., 119: 449, 1965.

135. MILLNER, A.J. and MILIS, I.H., J. Endocr., 47: 379, 1970.

136. BIRD, C.E., WIQVIST, N., DICZFALUSY, E., and SOLOMON, S., J. Clin. Endocr., 26: 1144, 1966.

137. PASQUALINI, J.R., WIQVIST, N. and DICZFALUSY, E., Biochim. Biophys., Acta. 121: 430, 1966.

138. BIRD, C.E., SOLOMON, S., WIQVIST, N. and DICZFALUSY, E., Biochim. Biophys. Acta. 104: 623, 1965.

139. COLAS, A., HEINRICHS, LER, W. and TATUM, H.J., Steroids, 3: 417, 1964.

140. SIMMER, H.H., EASTERLING, Jr., W.E., PION, R. and DIGNAM, W.J., Steroids, 4: 125, 1964.

141. JAFFE, R.B., LAMONT, K.G. and PEREZ-PALACIOS, G., In: Abstracts, III Inter--national Symposium of Endocrinology, Mexico, D.F. 1968, p. 171. Editor: C.Gual, ICS 157, Excerpta Medica, Amsterdam.

142. SHIMIZU, K., SHIMAO, S. and TANAKA, M., Steroids, 5: Suppl. 1, 85, 1965.

143. BLOCH, E., TISSENBAUM, B., RUBIN, B.L. and DEANE, H.W., Endocrinology, 71: 629, 1962.

144. ARCHER, D.F., WU, C., FLICKINGER, G.L. and TOUCHSTONE, J.C., Am. J. Obst. Gynec., 105: 972, 1969.

145. SHIRLEY, I.M. and COOKE, B.A., J. Endocr., 44: 411, 1969.

146. BOLTE, E., WIQVIST, N. AND DICZFALUSY, E., Acta. Endocr. (kbh), 52: 583, 1966.

147. SLAUNWHITE, W.R., KARSAY, M.A., HOLLMER, A. SANDBERG, A.A. and NISWANDER, K., Steroids, 6: Suppl. 11, 211, 1965.

148. SHACKLETON, C.H.L., KELLY, R.W., ADHIKARY, P.M., BROOKS, C.J.W., HARKNESS, R.A., SYKES, P.J. and MITCHELL, F.L., Steroids, 12: 705, 1968.

149. NAKAYAMA, T., ARAI, K., SATOH, K.,YANAIHARA, T., NAGATOMI, K. and FUJITA, Y., In: Abstracts, III International Congress of Endocrinology, Mexico, D.F., 1968. p. 171.

150. KIRSCHNER, M.A., WIQVIST, N. and DICZFALUSY, E., Acta. Endocr. (Kbh), 53: 584, 1966.

151. WENGLE, B., Acta. Soc. Med. Upsalien, 69: 105, 1964.

152. KNAPSTEIN, P., DAVID, A., WU, C.-H., ARCHER, D.F., FLICKINGER, G.L. and TOUCHSTONE, J.C., Steroids, 11: 885, 1968.

153. SHACKLETON, C.H.L., LIVINGSTONE, J.R.B. and MITCHELL, F.L., Steroids, 11: 299, 1968.

154. BERTRAND, J., SAEZ, J.M. In: Abstracts, International Symposium on Feto-placental Unit, Milan, 1968, p. 7. Editors: A.Pecile and G.B.Carru--thers, ICS 170, Excerpta Medica, Amsterdam.

155. HAYNES, Jr., R.C., MIKHAIL, G., ERIKSSON, G., WIQVIST, N. and DICZFALUSY, E., Acta. endocr. (Kbh), 45: 297, 1964.

156. SCHWERS, J., ERIKSSON, G. and DICZFALUSY, E., Acta. Endocr. (Kbh), 49: 65, 1965.

157. SCHWERS, J., GOVAERTS-VIDETSKY, M., WIQVIST, N. and DICZFALUSY, E., Acta. Endocr. (Kbh), 50: 597, 1965.

158. EMERMAN, S., DANCIS, J., LEVITZ, M., WIQVIST, N. and DICZFALUSY, E., J. Clin. Endocr., 25: 639, 1965.

159. ZUCCONI, G., GOEBELSMANN, U., WIQVIST, N. and DICZFALUSY, E., Acta. Endocr., 56: 71, 1967.

160. SCHWERS, J., ERIKSSON, G., WIQVIST, N. and DICZFALUSY, E., Biochim. Biophys., Acta. 100: 313, 1965.

161. KNUPPEN, R., BREUER, H. and DICZFALUSY, E., In: Abstracts, II Inter--national Congress on Hormonal Steroids, Milan, 1966, p. 171. Editors: E.B.Romanoff and L.Martini, ICS III, Excerpta Medica, Amsterdam.

162. DICZFALUSY, E. and MAGNUSSON, A.M., Acta. Endocr. (Kbh), 28: 169, 1958.

163. DICZFALUSY, E., MENINI, E., TILLINGER, K.G. and WESTMAN, A., Acta. Endocr. (Kbh), 30: 539, 1959.

164. DICZFALUSY, E., TILLINGER, K.G. and WESTMAN, A., Acta. Endocr.(Kbh), 26: 303, 1957.

165. DICZFALUSY, E., TILLINGER, K.G. and WESTMAN, A., Acta. Endocr. (Kbh), 26: 313, 1957.

167. ANGEVINE, D.M., Arch. Pathol., 26: 507, 1938.

166. LANMAN, J.T., In: "The Human Adrenal Cortex" (A.R.Currie, T.Symington and J.K.Grant, eds.), p. 547, 1962. Livingstone, Edinburgh and London.

168. KEENE, M.F.L. and HEWER, E.E., J. Anat., 61: 302, 1927.

169. BENIRSCHKE, K., Obstet. Gynecol., 8: 412, 1956.

170. TAYLOR, N.R.W., LORAINE, J.A. and ROBERTSON, H.A., J. Endocrinol. 9: 334, 1953.

171. BERSON, S.A.and YALOW, R.S., J. Clin. Invest., 47: 2725, 1968.

172. JOHANNISSON, E., Acta. Endocrinol(Copenhagen), Suppl. 130: , 1968.

173. MEYER, R., Virchow's Arch. Pathol. Anat. Physiol. 210: 158, 1912.

174. KIYONO, H., Virchow's Arch. Pathol. Anat. Physiol. 257: 441, 1925.

175. BENIRSCHKE, K., Obstet. Gynecol., 8: 412, 1956.

176. ROTTER, W., Virchow's Arch. Pathol. Anat., 316: 590, 1949.

177. JONES. I.C., Brit. Med. Bull., 11: 156, 1955.

178. LANMAN, J.T. and DINERSTEIN, J., Endocrin. 64: 494, 1959.

179. BURNER, J.A. J.Clin. Endocr. 11: 360, 1951.

180. LAURITZAN, C., In: "Research on Steroids", p. 109, Transactions of the Se--cond Meeting of the International Study Group for Steroid Hormones, Rome, Dec. 1965.

181. LAURITZEN, C. and LEHMANN, W.D., J. Endocr., 39: 173, 1967.

182. GISELA de RIDDER, M.D. THESIS, University of Kiel, 1969.

183. FRIEDRICHS, M. University of Kiel, 1968.

184. GREEP, R.O., VANDYKE, H.D. and CHOW, B.,F. Proc. Soc. Exp. Biol. Med., 46: 644, 1941.

185. GEIGER, W., KAISER, R. and FRANCHIMONT, P. Acta, Endocr., 68: 169, 1971.

186. LAURITZEN, C. and LEHMANN, W.D. Arch. Gynak. , 200: 699, 1965.

187. LAURITZEN, C., SHACKLETON, C.H.L. and MITCHELL, F.L. Acta Endocr. (Kbh), 58: 655, 1968.

188. BRODY, S. and CARLSTROM, G. J. Clin. Endocr., 25: 792, 1965.

189. CASSMER, O. Acta. Endocr. (Kbh), Suppl. 45: 1, 1959.

190. DORFMAN, R.I. and VAN WAGENEN, G. Surg. Gyneco. and Obstet. 73: 545, 1941.

191. KNOWLTON, A.I., MUDGE, G.H. and JAILER, J.W. J.Clin. Endocr. 9: 514, 1949.

192. LITTLE, B., SMITH, O.W., JESSIMAN, A.G., SELENKOW, H.A., VANT HOFF, W., ENGLIN, J.M. and MOORE, F.D. J.Clin. Endocrinol. Metabo., 18: 425, 1958.

193. FRANDSEN, V.A. and STAKEMANN, G. Acta. Endocr. (Kbh), 38: 383, 1961.

194. FRANDSEN, V.A. and STAKEMANN, G. Acta. Endocr. (Kbh), 47: 265, 1964.

195. FRANDSEN, V.A. and STAKEMANN, G. Acta. Endocr. (Kbh), Suppl. 90: 81, 1964.

196. FRANDSEN, V.A. and STAKEMANN, G. Acta. Endocr. (Kbh), 43: 184, 1963.

197. MANCUSO, S., NILSSON, L. and DICZFALUSY, E., In: Research on Steroids, Vol. 2, p.47, 1966. Editor: C.Cassano, II Pensiero, Scientifico,Rome.

198. BAULIEU, E.E. and DRAY, F. J. Clin. Endocr., 23: 1298, 1963.

199. SIITERI, P.K. and Mac DONALD, P.C. Steroids, 2: 713, 1963.

200. WARREN, J.C. and TIMBERLAKE, C.E. Obstet. Gynec. 23: 689, 1964.

201. BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. and DICZFALUSY, E. Acta. Endocr. (Kbh), 45: 576, 1964.

202. MANCUSO, S., MANCUSO, F.P., TILLINGER, K.G. and DICZFALUSY, E. Acta. Endocr. (Kbh), 49: 248, 1965.

203. DELL'ACQUA, S., MANCUSO, S., ERIKSSON, G. and DICZFALUSY, E. Acta. Endocr. (Kbh), Suppl. 100: 81, 1965.

204. DELL'ACQUA, S., MANCUSO, S., WIQVIST, N. , RUSE, J.L., SOLOMON, S. and DICZFALUSY, E. Acta. Endocr. (Kbh), 55: 389, 1967.

205. MIKHAIL, G. and ALLEN, W.M. Am. J. Obstet. Gynecol. 99: 308, 1967.

206. YOSHIMI, T., STROTT, C.A., MARSHALL, J.R. and LIPSETT, M.B. J. Clin. Endocr. Metab. 29: 225, 1969.

207. ASK-UPMARK, M.E. Acta. Obstet. Gynecol. Scand., 5: 211, 1926.

208. TULSKY, A.S. and KOFF, A.K. Fert. Steril., 118, 1957.

209. DICZFALUSY, E. and BORELL, U. J. Clin. Endocr. Metab., 21: 1119, 1961.

210. REBBE, H. and MOLLER, K.J.A. Acta. Obstet. Gynecol. Scand. 45: 261, 1966.

211. HYTTEN, F.E. and LEITCH, I. In: Physiology of Human Pregnancy", p. 155, 1964, Blackwell, Oxford.

212. KLOPPER, A.I. Obstet. Gynecol. Surv., 23: 813, 1968.

213. BERNISCHKE, K. and DRISCOLL, S.G. In: "The Pathology of Human Placenta", p. 446, 1967, Springer, Berlin.

214. MILIC, A.B. and ADAMSONS, K. J.Obstet. Gynecol., 76: 102, 1969.

215. UTTLEY, W.S. Arch. Dis. Childhood, 43: 724, 1968.

216. O'DONOHOE, N.V. and HOLLAND, P.D.J. Arch. Dis. Childhood, 43:717, 1968.

217. FRANCE, T.J. and LIGGINS, G.C. J.Clin.Endocrinol. Metab., 29: 138, 1969.

218. CSAPO, A.I. and WOOD, C. In: "Recent Advances in Endocrinology" (V.H.T. James, ed.), p. 207, Churchill, London.

219. VILLEE, C.A., HAGERMANN, D.D. and JOEL, P.B. Recent Progr. Hormone Res. 16: 49, 1960.

220. TALALAY, P. and WILLIAMS-ASHMAN, H.H. Recent Progr. Hormone Res. 16: 1, 1960.

221. SZECO, C.M. FEDERATION Proc. 24: 1343, 1965.

222. BAULIEU, E.E. In: "Research on Steroids"(C.Cassano ed.,) Vol II, Pensiero Sci., Rome.

223. KARLSON,P. ed., "Mechanism of Hormone Action", Academic Press, New York.

224. OAKEY, R.E. Vitamins and Hormones, 28: 1, 1970.

225. HEINRICHS, W.L. FEDER, H.H. and COLAS, A. Steroids, 7: 91, 1966.

226. SIMMER, H.H., DIGNAM, W.J., EASTERLING, W.E., FRANKLAND, M.V. and NAFTOLIN, F. Steroids, 8: 179, 1966.

227. WRAY, P.M. and RUSSELL,C.S. J.Obstet. Gynecol. Brit.Commonw.71: 97, 1964.

228. WALLACE, S.J. and MICHIE, E.A. Lancet, 560, 1966.

229. OAKEY, R.E., BRADSHAW, L.R.A., ECCLES, S.S., STITCH, S.R. and HEYS, R.F. Clin.Chim. Acta. 15: 35, 1967.

230. SCOMMEGNA, A., NEDOSS, B.R. and CHATTORAJ, S.C. Obstet. Gynecol., 31: 526, 1968.

231. DRISCOLL, A.M. Brit. Med. J., 1: 556, 1969.

232. DASSLER, C.G. Acta. Endocrinol. (Copanhagen), 53: 401, 1966.

233. MAEYMA, M., NAKAGAWA, T., TUCHIDA, Y., and MATUOKA, H. Steroids, 13: 59, 1969.

234. LIDDLE, G.W., ESTEP, H.L., KENDALL, J.W., WILLIAMS, W.C. and TOWNES, A.W. J. Clin. Endocrinol. Metab., 19: 875, 1959.

235. DICKEY, R.P. and THOMPSON, J.P. J. Clin. Endocrinol. Metab. 29: 701, 1969.

236. OAKEY, R.E. and HEYS, R.F. J. Endocrinol., 45: XXIII, 1969.

237. OAKEY, R.E. and HEYS, R.F. Acta. Endocrinol., 65: 502, 1970.

238. SANDBERG, A.A. and SLAUNWHITE, W.R. J. Clin. Invest., 38: 1290, 1959.

239. DE MOOR, P., HEIRWEGH, K., HEREMANS, J.F. and DECLERCK-RASKIN, M. J. Clin. Invest. 41: 816, 1962.

240. MILLS, I.H., CHEN, P.S. and BARTLER, F.C. J. Endocrinol. 18: XXX, 1959.

241. DAUGHADAY, W.H., KOZAK, I. and BIEDERMAN, O. J. Clin. Invest, 38: 998, 1959.

242. GREIG, M., COYLE, M.G., COOPER, W. and WALKER, J. J. Obstet. Gynecol. Brit. Commonw., 69: 772, 1969.

243. MURPHY, B.E.P. J. Clin. Endocrinol. Metab. 27: 973, 1967.

244. ROSENTHAL, H.E., SLAUNWHITE, W.R. and SANDBERG, A.A. J. Clin. Endocrinol. metab., 29: 352, 1969.

245. MIGEON, C.J., PRYSTOWSKY, H., GRUMBACH, M.M. and BYRON, M.C. J. Clin. Invest., 35: 488, 1956.

246. AARSKOG, D. Acta. Paediat. Scand., Suppl. 158, 1965.

247. MIGEON, C.J., KENNY, F.M. and TAYLOR, F.H., J. Clin. Endocrinol. Metab. 28: 661, 1968.

248. LANDON, J., JAMES, V.H.T, and PEART, W.S. Acta. Endocrinol. (Copenhagen) 56: 321, 1967.

249. STONE, D. and HECHLER, O. Arch. Biochem. Biophys. 51: 457, 1954.

250. BORKOWSKI, A.J., LEVIN, S., DELCROIX, C., MAHLER, A. and VERHAS, V. J. Clin. Invest. 46; 797, 1967.

251. WHITEHOUSE, B.J. and VINSON, G.P. Steroids, 11: 245, 1968.

252. VILLEE, D.B. Advan. Enzyme. Regul. 4: 269, 1966.

254. SYNDOR, K.L., KELLEY, V.C., RAILE, R.B., ELY, R.S. and SAYERS, G. Proc. Soc. Exp. Biol. Med., 82: 695, 1953.

253. TAHKA, H. Acta. PAEDIAT. Scand. 40: Suppl. 81, 1951.

255. BLACKMAN, S.S., Bull. Johns Hopkin Hosp. 78: 180, 1946.

256. PAUERSTEIN, C.J. and SOLOMON, D. Obstet. Gynecol. 28: 692, 1966.

257. UMBREIT, W.W., BURRIS, R.H., STAUFFER, J.F. Manometric Techniques, Burgen publishing co., Minneapolis, p. 149, 1957.

258. KLIMAN, B. and PETERSON, R.E. J. Biol. Chem., 235: 1639, 1960.

259. BUSH, I.E. The Chromatography of Steroids, Pergamon Press, New York, p. 347, 1961.

260. LISBOA, B.P. Steroids, 6: 605, 1965.

261. TSANG, C.P.W. Ph.D. Thesis, McGill University, Montreal, 1968.

262. DIAB, I.M. and COMALL, A.W. Steroids, 8: 109, 1966.

263. DURNHOFFER, F. M.Sc. Thesis, McGill University, Montreal, 1969.

264. TRULS BRINCK-JOHNSEN, Acta. Endocrinol. Vol. 3, 696, 1970.

265. BUSH, I.E. Biochem. J. 50: 370, 1952.

266. EBERLEIN, W.R. and BONGIOVANNI, A.M. Arch. Biochem. 59: 90, 1955.

267. BAULIEU, E.E. J. Clin. Endocrinol, Metab. 20: 900, 1960.

268. BUSH, I.E. The Chromatography of Steroids, Pergamon Press, New York, p. 378, 1961.

269. AXELROD, L.R., MATTHIJSSCN, C., GOLDZIEHER, W. and PULLIAM, J.E. Acta. Endocrinol. Suppl. 99: 7, 1965.

270. OKITA, G.I., KABARA, J.J., RICHARDSON, F. and LEROY, G.V., Nucleonics, 15; 111, 1957.

271. STACHENKO, J., LAPLANTE, C. and GIROUD, C.J.P., Can. J. Biochem., 42: 1275, 1964.

272. DOUNCE, A.L., WITTER,R.F., MONTY, K.J., PATE, S. and COTTONE, M.A., J. Biophysic. Biochem. Cytol., Vol. 1: No. 2, 139, 1955.

273. GIROUD, C.J.P., STACHENKO, J. and VENNING, E.H., Proc. Soc. Expt. Biol.& Med., 92: 154, 1956.

274. LEE, D.S.C., M.Sc. Thesis, McGill University, Montreal, 1970.

275. STACHENKO, J., LEE, D.S.C., and NAGY, E., Steroids; 17: 2, 175, 1971.

276. DICZFALUSY, E. and BORELL, M.D., J. Clin. Endocr. and Metab, 21:1119, 1961.

277. SCHMIDT, E.H., and SEUKEN, A.S.W., Acta Endocrinol. Suppl. 100: 108, 1965.

278. SUTHERLAND, E.W. and RALL, T.W., Pharmacol. Revs. 12: 265, 1960.

279. BDOLAH, A. and SCHRAMM, M., Biochem. Biophys. Res. Comm. 18: 452, 1965.

280. PION, R.J., JAFFE, R.B., WIQVIST, N. and DICZFALUSY, E., Biochem. Biophys. Acta (Amst.) 137: 584, 1967.

281. CEDARD, L., ALSAT, E., EGO, C. and VARANGOT, J., Steroids, 11: 2,179, 1968.

282. PINCUS, G., In: Transactions of the Third Conference on Getstion, New York, Josiah Macy, Jr., Foundation, p. 91, 1957.

283. SAURE, A., 5th. Acta Endocrinologica Congress, Abst. No. 11, Acta Endocr. Suppl. 100: 43, 1965.

284. STERN, M.D., M.Sc. Thesis, McGill University, Montreal, 1968.

285. HAYNES, Jr., R.C., SUTHERLAND, E.W. and RALL, T.W.,Recent Progr. Hormone Res. 16: 121, 1960.

286. MARSH, J.M., BUTCHER, R.W., SAVARD, K. and SUTHERLAND, E.W., J. Biol.Chem. 241: 5436, 1966.

287. KOWAL, J. Recent Prog. Hormone Res. 26: 623, 1970.

288. HAYANO, M., SABA, N., DORFMAN, R.I. and HETCHER, O., Recent Prog. in Hormone Res. XII: 79, 1956.

289. KHANT, F.W. and NEHER, R., Hevl. Chim. Acta 48: 1457, 1965.

290. MACCHI, I.A. and HECHTER, O., Arch. Biochem. and Biophys. 53: 305, 1954.

291. REICH, E. and LEHNINGER, A.L., Biochim et Biophys. Acta 17: 136, 1955.

292. SAFFRAN, M., GRAD, B. and BAYLISS, M.J., Endocrinol. 50: 639, 1953.

293. LONGCHAMPT, J.E., GUAL, C., EHRENSTEIN, M. and DORFMAN, R.I., Endocrinology 66: 416, 1960.

294. MORATO, T., HAYANO, M., DORFMAN, R.I. and AXELROD, L.R., Biochem. Biophys. Res. Commun. 6: 334, 1961.

295. GUAL, C., MORATO, C., HAYANO, M., GUT, M. and DORFMAN, R.I., Endocrinology, 71: 920, 1962.

296. MORATO, T., RAAB, K., BRODIE, H.J., HAYANO, M. and DORFMAN, R.I., J. Amer. Chem. Soc. 84: 3764, 1962.

297. WILCOX, R.B. and ENGEL, L.L., Steroids, Suppl. 1: 49, 1965.

298. AKHTAR, M. and SKINNER, S.J.M., Biochem. J. 109: 318, 1968.

299. TOWNSLEY, J.D. and BRODIE, H.J., Biochemistry, 7: 33, 1968.

300. SHAW, D.A., DALZIEL, E.M. and O'DONNELL, V.J., Biochem. J. 113: 737, 1969.

301. HECHTER, O., ZAFFARONI, A., JACOBSEN, R.P., LEVY, H., JEANLOZ, R.W., SCHENKER, V. and PINCUS, G., Recent Prog. Horm. Res. 6: 215, 1951.

302. HAIKERSION, I.D.K., EICHHORN, J. and HECHTER, O., J. Biol.Chem. 236: 374, 1961.

303. McKERNS, K.W., Biochim. Biophys. Acta 90: 357, 1964.

304. VILLEE, C.A. and LORING, J.M., J. Clin. Endocr. Metb. 25: 307, 1965.

305. BAILLIE, A.H., NIEMI, M. and IKONEN, M., Acta Endocr. 48: 429, 1965.

306. NIEMI, M. and BAILLIE, A.H., Acta Endocr. 48: 423, 1965.

307. JONES, H.W. and JONES, G.S., Editor's note, Obstet. Gynec. Survey, 20: 636, 1965.

308. MITCHELL, E.L., Vitamins and Hormones, 25: 191, 1967.

309. GOLDMAN, A.S., BONGIOVANNI, A.M., YAKOVAC, W.C. and PRADER, A., J.Clin. Endocrinol. Metab. 24: 894, 1964.

310. GOLDMAN, A.S., YAKOVAC, W.C. and BONGIOVANNI, A.M., J.Clin. Endocr.Metab. 26: 14, 1966.

311. CAVALLERO, C. and MAGRINI, U., Excerpta Med.Intern. Congr. Ser. 111:63, 1966.

312. GRIFFITHS,K.J., Endocrinol. 26: 437, 1953.

313. SAMUELS, L.T., HELMREICH, M.L., LASATER, M.B. and REICH, H., Science, 113: 490, 1951.

314. KOWAL, J., FORCHIELLI, E. and DORFMAN, R.I., Steroids, 3: 531, 1964.

315. KOWAL, J., FORCHIELLI, E. and DORFMAN, R.I., Steroids, 4: 77, 1964.

316. NICOLIS, G.L. and ULICK, S., Endocrinol, 76: 514, 1965.

317. KESWANI, I., M.Sc. Thesis, McGill University, Montreal, 1969.

318. EVANS, H.M. and SIMPSON, M.E., "The Hormones", Vol. II(Academic PressInc. New York) p.351, 1950.

319. BRADY, R.O., J.Biol. Chem. 193: 145, 1951.

320. LAATIKAINEN, T., LAITINEN, E.A. and VIHKO, R., J.of Clin. Endocrinol. and Metab. 32: 59, 1971.

321. SAMUELS, L.T.and HELMREICH, M.L., Endocrinology, 58: 435, 1956.

322. HAYANO, M., LONGCHAMPT, J.E., KELLY, W., GUAL, C. and DORFMAN, R.I., Acta Endocrinol. Suppl. 50: 699, 1960.

323. HOLLANDER, N., Endocrinology, 71: 723, 1962.

324. SUTHERLAND, E.W., Harvey Lect. 57: 17, 1961.

325. LEFKOWITZ, R.J., ROTH, J., PRICER, W. and PASTAN, I., Proc. Nat. Acad. Sci. U.S. 65: 745, 1970.

326. SCHIMMER, B.P., UEDA, K. and SATO, G.H., Biochem. Biophys. Res. Commun. 32: 806, 1968.

327. GARREN, L.D., GILL, G.N., MASUI, H. and WALTON, G.M., Rec. Prog. In. Horm. Res. 27: 433, 1971.

328. VENNING, E. and SIBULSKI, S., In: "Meeting on Biological and Clinical Aspects of Placental Steroidogenesis"(F.Polvani and A.Bompiani, Eds.), p. 13-21, Williams and Wilkins, Baltimore, Maryland, 1964.

329. GALLAGHER, T.F., Proceedings of the First International Congress of Hormonal Steroids, (Vol. 1), Acad. Press. New York, 1965, p.103.

330. GEIST, S.H., Amer. J. Obstet. Gynec. 26: 588, 1933.

331. WESTMAN, A., Acta Obstet. et Gynec. Scand. 17: 492, 1937.

332. ARCHER, D.F., MATHUR, R.S., WIQVIST, N. and DICZFALUSY, E., Acta Endocr. 66: 666, 1971.