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STUDY OF PROGESTERONE PRODUCTION IN HUMAN PREGNANCY BY EARLY PLACENTAL EXPLANTS

by

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In memory of my late father for his immortal academic inspiration and encouragement for higher studies.

ABSTRACT

Little is known about the factors stimulating placental progesterone (P4) production at the time of the luteo-placental shift (6 - 8 weeks post-conception). To explore the regulatory mechanism, the effects of various steroids and peptides on the production of P4 by placental explants were studied.

In early placental explant culture P4 production was stimulated by 19-nortestosterone (19-NT), androstenedione (A-dione), 5α-androstane-3α,17β diol (3α-diol) and 5α-androstane-3β,17β diol (3β-diol). Of all the compounds tested, 19-NT had maximal effect. At term, P4 production was stimulated only by 3β-diol. 19-NT and A-dione were poorly aromatized in early placental explants compared to another androgen (Androst-5-ene-3β, 17β diol).

In accord with the above observations, placental levels of 19-NT and A-dione were higher in early gestation while the diols were higher in late gestation.

19-NT stimulated P4 production in early placenta by effects on the conversion of P4 both from 25-hydroxycholesterol and from pregnenolone. The stimulatory influences of A-dione and 3α-diol were mediated by increasing the P450scc activity. The specific increase of the conversion of P4 from pregnenolone accounted for the P4 stimulation observed by 3β- diol treatment of culture.

Cyloheximide (CH) treatment abolished the stimulatory influences of the aforementioned steroids on P4 production except for the initial phase of P4 stimulation by 19-NT, suggesting that all but the latter are dependent on protein synthesis.

P4 production was also stimulated and prolonged to 30 days in the presence of human maternal serum (HMS) ; a good correlation ($r = 0.74$, $P < 0.05$) was seen between the histological appearance of the explants and P4 production. The stimulatory activity of HMS was heat labile, non-dialyzable and non-extractable into an organic solvent, suggesting that it is protein in nature.

In conclusion, this study suggests that 19-NT and A-dione are important for placental P4 production at the time of the luteo-placental shift. For in vitro study of placental hormonal regulation, HMS is a better nutrient supplement than fetal bovine serum.

RESUME

Les facteurs stimulant la production de la progestérone placentaire (P4) au moment de la transition lutéo-placentaire (6 - 8 semaines après la conception) sont peu connus. Afin de savoir quels sont les mécanismes régulateurs, on a étudié l'effet des différents stéroïdes et peptides sur la production de P4 par les explants placentaires obtenues à cette étape du développement.

On a stimulé la production de P4 avec la 19-nortestostérone (19-NT), l'androstènedione (A-dione), le 5α-androstane-3α,17β-diol (3α-diol) et le 5α-androstane-3β,17β-diol (3β-diol). La stimulation maximale a été obtenue avec la 19-NT. A terme, la production de P4 ne fut stimulée que par le 3β-diol. La 19-NT et l'A-dione étaient faiblement aromatisés dans les explants placentaires, lorsque comparés à un autre androgène, l'androst-5-ène-3β,17β-diol.

Conformément aux observations susmentionnées, les niveaux de 19-NT et d'A-dione placentaires étaient plus élévés en début de gestation, alors que ceux des diols étaient plus élévés à la fin.

Au début de gestation, la 19-NT a stimulé la production de P4 en agissant sur sur la conversion du 25-hydroxycholestérol et du prégnénolone en P4. L'effet stimulateur de l'A-dione et du 3α-diol a été obtenu par l'augmentation de l'activitié du cytochrome P_{450}scc. Quant à la stimulation de P4 observée après administration de 3β-diol, elle s'explique par la conversion de prégnénolone en P4.

Le traitement à la cycloheximide a aboli l'effet stimulateur des stéroïdes susmentionnés sur la production de P4, sauf pendant la phase initiale de stimulation par le 19-NT. Ceci suggère qu'ils dépendent tous, à l'exception de ce dernier, de la synthèse protéique.

La production de P4 a aussi été stimulée et prolongée jusqu'à 30 jours en présence de sérum humain maternel. Il existait une corrélation satisfaisante ($r = 0.74$, $P \leq 0.05$) entre l'apparence histologique des explants et la production de P4. Le facteur stimulateur du sérum était thermolabile, non dialysable et non extractible dans un solvant organique, suggérant ainsi qu'il est de nature protéique.

En conclusion, l'étude suggère que le 19-NT et l'A-dione jouent un rôle important dans la production du P4 placentaire au moment de la transition lutéo-placentaire. Dans les études *in vitro* sur la régulation hormonale placentaire, le sérum humain maternel est un meilleur supplément nutritif que le sérum bovin fétal.

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GLOSSARY OF TERMS

^{3}H	radioactive isotope of hydrogen, atomic weight 3
^{125}I	radioactive isotope of iodine, atomic weight 125
Androstenedione	4-Androstene-3,17-dione
Dehydroepiandrosterone	3ß-Hydroxy-5-androsten-17-one
Dehydroepiandrosterone sulfate	17-Oxo-5-androsten-3ß-yl-sulfate
Dexamethasone	9α-Fluoro-16α-methyl-11ß,17α,21-trihydropregna-1,4-diene-3,20-dione
5α-Dihydroprogesterone	5α-Pregnane-3,20-dione
20α-Dihydroprogesterone	20α-Hydroxy-4-pregnen-3-one
5α-Dihydrotestosterone	17ß-Hydroxy-5α-androstan-3-one
Estradiol	1,3,5(10)-Estratriene-3,17ß-diol
Estriol	1,3,5(10)-Estratriene-3,16α,17ß-triol
Estrone	3-Hyrdoxy-1,3,5(10)-estratriene-17-one
17α-Hydroxyprogesterone	17α-Hydroxy-4-pregnene-3,20-dione
19-Nortestosterone	17ß-Hydroxy-4-estren-3-one
Pregnenolone	3ß-Hydroxy-5-pregnen-20-one
Progesterone	4-Pregnene-3,20-dione
Testosterone	17ß-Hydroxy-4-androsten-3-one

LIST OF ABBREVIATIONS

Ab	antibody
ACTH	adrenocorticotropin
A-dione	androstenedione
Ag	antigen
^0C	degree Celsius
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CH	cycloheximide
CO_2	carbon dioxide
CPM	counts per minute
CPB	competitive protein binding
CV	coefficient of variation
dbcAMP	dibutyrl cyclic adenosine monophosphate
DCC	dextran coated charcoal
DHA	dehydroepiandrosterone
DHAS	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
3α-diol	5α-androstane-3α,17β diol
3β-diol	5α-androstane-3β,17β diol
E_1	estrone
E_2	estradiol
EGF	epidermal growth factor
F	cortisol
FBS	fetal bovine serum
FSH	follicle stimulating hormone
GIBCO	Grand Island Biological Company
GH	growth hormone
gm	gram
GnRH	gonadotropin releasing hormone
hr	hour
hCG	human chorionic gonadotropin
HDL	high density lipoprotein
HLA	human leukocyte antigen(s)
HMS	human maternal serum
HSD	hydroxysteroid dehydrogenase
Ig	immunoglobulin
IU	international unit
K_D	equillibrium dissociation constant
kg	kilogram
L	litre
LDL	low density lipoprotein
LMP	last menstrual period
LCS	liquid scintillation counter
M	molar
mM	millimolar
mm	millimeter
mL	millilitre

mg	milligram
mCi	millicurie
MHC	major histocompatibility complex
min	minutes
mRNA	messenger ribonucleic acid
MW	moleculer weight
μ (10^{-6})	microns
μg	microgram
μCi	microcurie
μM	micromolar
n	number of observation
ng (10^{-9}gm)	nanogram(s)
nm	nanometer
nM	nanomolar
NAD	nicitinamide adenine dinucleotide
19-NT	19-nortestosterone
O_2	oxygen
OH	hydroxy
17α-OHP	17α-hydroxyprogesterone
25-OHC	25-hydroxycholesterol
P	probability
%	percentage
pg (10^{-12}gm)	picogram(s)
P4	progesterone
P5	pregnenolone
PBG	progesterone-binding globulin
r	correlation coefficient
rpm	revolutions per minute
RIA	radioimmunoassay
RTA	radiotransinassay
SA	specific activity
SD	standard deviation
SE	standard error
SHBG	sex hormone-binding globulin
T	testosterone
TE	testosterone equivalent
wks	weeks
wt.	weight

TABLE OF CONTENTS

		Page
ABSTRACT		i
RESUME		ii
ACKNOWLEDGEMENTS		iv
GLOSSARY OF TERMS		v
LIST OF ABBREVIATIONS		vi
TABLE OF CONTENTS		viii
LIST OF FIGURES		xi
LIST OF TABLES		xv
PREFACE		1
CHAPTER I	HISTORICAL REVIEW OF THE HUMAN PLACENTA	
1.1	Introduction	6
1.2	Embryonic Development	6
1.3	Gross Anatomy and Histology	9
1.4	Immunological Aspects	10
1.5	Clinical Aspects	11
1.6	Nutrient Transport and Gas Exchange	12
1.7	Endocrine Aspects	16
1.7.1	Human Chronic Gonadotropin (hCG)	17
1.7.2	Human Placental Lactogen (hPL)	18
1.7.3	Androgens	19
1.7.4	Estrogens	21
1.7.5	Progesterone	23
1.8	Tissue Culture	38
1.9	Purpose of the Study	43
1.10	References for Chapter I	45
CHAPTER II	EXPERIMENTAL	
2.1	Materials	64
2.2	Tissue Culture	66
2.2.1	Aseptic Technique	66
2.2.2	Antibiotics for Culture Medium	67
2.2.3	Medium Preparation	67
2.2.4	Explant Culture	68
2.3	Extraction Procedure	72

Page

2.4 Column Chromatography 72

2.4.1 General technique of Chromatography 72
2.4.2 Counting of Radioactivity 76

2.5 Hormone Analysis 76

2.5.1 Buffer Solutions 76
2.5.2 Charcoal Solution 78
2.5.3 Steroid Solution 78
2.5.4 Protein Binding Assay 79
2.5.4.1 RTA for Progesterone . 80
2.5.4.2 RIA for Progesterone . 82
2.5.4.3 RTA for SHBG 83
2.5.4.4 RIA for 19-Nortestosterone 83
2.5.4.5 RIA for Androstenedione 84
2.5.4.6 RIA for 17β-Estradiol . 84
2.5.4.7 RIA for Estrone 85
2.5.4.8 RIA for β-hCG 85
2.5.4.9 Reliability Criteria . 87

2.6 References for Chapter II 99

CHAPTER

III IN VITRO STIMULATION OF PROGESTERONE BY 19-NORTESTOSTERONE AND C_{19} STEROIDS IN EARLY HUMAN PREGNANCY

3.1 Abstract 103
3.2 Introduction 104
3.3 Materials and Methods 106
3.4 Results 111
3.5 Discussion 117
3.6 References 133

CHAPTER

IV CONCENTRATIONS OF 19-NORTESTOSTERONE AND SOME ANDROGENS IN HUMAN PLACENTA.

4.1 Abstract 140
4.2 Introduction 141
4.3 Materials and Methods 141
4.4 Results 146
4.5 Discussion 148
4.6 References 159

CHAPTER Page

V INFLUENCE OF 19-NORTESTOSTERONE AND ANDROGENS ON PROGESTERONE BIOSYNTHETIC ENZYMES IN EARLY HUMAN PLACENTAL EXPLANTS.

5.1 Abstract 166
5.2 Introduction 168
5.3 Materials and Methods 169
5.4 Results 174
5.5 Discussion 177
5.6 References 190

CHAPTER

VI EFFECT OF MATERNAL SERUM ON VIABILITY AND FUNCTION OF EARLY HUMAN PLACENTAL EXPLANTS.

6.1 Abstract 197
6.2 Introduction 198
6.3 Materials and Methods 199
6.4 Results 204
6.5 Discussion 208
6.6 References 236

CHAPTER

VII CLOSURE

7.1 Summary and Conclusions 240
7.2 Physiological Implications 244
7.3 Recommendations for Future Work 247
7.4 References for chapter VII 248

CHAPTER

VIII CONTRIBUTIONS TO KNOWLEDGE 250

APPENDICES

1. Formulation of the Ham's F-10 nutrient mixture. . 252
2. Specificity of the guinea pig serum. 254
3. Specificity of the progesterone antiserum (P11-192). 256
4. Specificity of the sex hormone-binding globulin. . 257
5. Specificity of the 19-nortestosterone antiserum. . 258
6. Specificity of the androstenedione antiserum. . . 259
7. Specificity of the estradiol antiserum. 260
8. Specificity of the monoclonal antibody for β-hCG. 261

LIST OF FIGURES

Figure		Page
2.1	A typical standard curve for the measurement of progesterone in the guinea pig assay	93
2.2	Example of a standard curve for the SHBG assay .	94
2.3	Example of a standard curve for the radioimmuno-assay for 19-nortestosterone	95
2.4	Example of a standard curve for androstenedione radioimmunoassay	96
2.5	Example of a standard curve for the radioimmuno-assay for estradiol	97
2.6	Example of a standard curve for the measurement of ß-hCG	98

CHAPTER III

3.1	Secretion of progesterone in control culture with the incubation time of 24 hours	127
3.2	Stimulation of progesterone by 5α-androstane-3ß,17ß diol and 5α-androstane-3α,17ß diol	128
3.3	Effect of increasing concentrations of androstenedione and 19-NT on progesterone formation in culture .	129
3.4	Time course of progesterone stimulation by 19-nortes-tosterone and androstenedione	130
3.5	Effect of 5α-androstane-3ß,17ß diol on term placental progesterone production.	131
3.6	Comparison of aromatization of androstenedione, 19-nortestosterone and androst-5-ene-3ß,17ß diol	132

CHAPTER IV

Figure Page

4.1 Concentrations after chromatography of A-dione 19-NT in placental pools 155

4.2 Immunoreactivity of 19-NT in unchromatographed samples of placental tissue through gestations . 156

4.3 Placental levels of androstanediol and androstenediol measured together, are compared at different gestational ages 157

4.4 The levels of testosterone and dihydrotestosterone in placental pools from early and term gestation. 158

CHAPTER V

5.1 Substrate-dependent conversion of 25-OHC to progesterone by placental explants incubated for 24 hours 186

5.2 Effect of 1 μM 19-NT, A-dione, 3α-diol and 3ß-diol on the conversion of exogenously added 25-OHC to progesterone in cultured media 187

5.3 Effect of cycloheximide (1 mM) on 19-NT (1 μM) treated cultures at different times of incubation . . . 188

5.4 Effect of 19-NT, A-dione, 3α- and 3ß-diol at 1 μM concentration, on the progesterone production in culture incubated for 24 hours in presence or absence of cycloheximide (1 mM) 189

CHAPTER VI

Figure		Page
6.1	Basal progesterone production in early placental culture with Ham's F-10 in presence or absence of FBS 10%	219
6.2	Basal progesterone production from early, midterm and term placental culture with Ham's F-10 + FBS 10%	220
6.3	Basal estradiol-17ß production from early and late placental culture in Ham's F-10 + FBS 10% . . .	221
6.4	Comparison of basal progesterone production by early placenta in presence of 10 or 40 % human maternal serum (HMS) and 10 or 40 % fetal bovine serum (FBS)	222
6.5	Comparison of estradiol-17ß production by early placenta in presence of 10 or 40 % FBS and 10 or 40% HMS.	223
6.6	The production of early placental ß-hCG in presence of 10 or 40% HMS and 10 or 40% FBS	224
6.7a	Early placental progesterone production in presence of FBS 10% or HMS 10% or in absence of serum. .	225
6.7b	Early placental estradiol-17ß production in presence of FBS 10% or HMS 10%.	226
6.7c	Early placental ß-hCG production in presence of FBS 10% or HMS 10%	227

Figure Page

6.8 Progesterone production by early placenta in the presence of treated HMS 10% 228

6.9 Early placental progesterone production in presence of FBS 10%, non-pregnant and pregnant human maternal serum from early (HMS) as well as from late (HMST) gestation 229

6.10 Progesterone production from early and term placental culture in presence of 10% FBS or HMS 10% 230

6.11 Progesterone production by early placenta in the presence 10% FBS, 10% HMS, 19-NT + 10% FBS or 19-NT + 10% HMS. 231

6.12 Fresh placental tissue before culture, from first trimester human pregnancy 232

6.13 Viability of early placental explants after 24 hours in culture under different conditions 233

6.14 Viability of early placental explants after seven days in culture under different conditions 234

6.15 Viability of early placental explants on different days in culture under different conditions . . . 235

LIST OF TABLES

CHAPTER II

Table		Page
2.1	Precision of explant preparation	71
2.2	Summary of hormone assays	90
2.3	Sensitivity of the PBG assay	87
2.4	Precision of the PBG assay	88
2.5	Performance characteristics of hormone assays .	92

CHAPTER III

3.1	Specificity of binding proteins used for assay, calculated at 50% displacement	124
3.2	Progesterone values determined before and after chromatography	125
3.3	Summary of the Effects of C_{18} and C_{19} steroids on estradiol and progesterone production in culture	125
3.4	Compounds without effect on progesterone production in culture	126
3.5	Early placental sulfatase activity	126

CHAPTER V

5.1	Biosynthesis of [^{3}H]-P4 from [^{3}H]-P5 by early human placental explant	185

CHAPTER VI

6.1	Tissue histology score	217
6.2	Comparison of the constituents of HMS and FBS .	218

PREFACE

According to the 'Guidelines Concerning Thesis Preparation (McGill University)', the research carried out for the thesis is written in the form of 'papers' suitable for submission to relevant journals for publications. The following is quoted directly from thesis guidelines.

> "The candidate has the option, subject to the approval of the Department, of including as part of the thesis, the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion".
>
> "It is acceptable for theses to include as chapters authentic copies of papers already published, provided

these are duplicated clearly on regular thesis stationary and bound as an integral part of the thesis. Photographs or other material which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary."

"The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of claims, e.g. before the Oral Committee. Since the task of Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before they submit the thesis for review."

Each of the manuscripts included in this thesis constitutes a chapter. In the interests of continuity and clarity, the manuscripts are presented sequentially as the work evolved. The references pertaining to a chapter are collected at the end of the chapter. Although relevant references and experimental methods are mentioned briefly in each manuscript, detailed descriptions of methodologies and a general review of placenta along with tissue culture are included in the thesis for a clear understanding of the importance of this work.

Due to the presentation of the thesis in the form of

manuscripts, a reference and parts of text may appear more than once. This was necessary for the coherence and completeness of each manuscript.

I am the first and principal author of the manuscripts presented in chapters 3, 4, 5 and 6. All four manuscripts were co-authored by Dr. Beverley Pearson Murphy, my research supervisor; the 2nd (chapter 4) was co-authored as well by Drs. Charlotte Branchaud and Peter Gillette who provided placental samples, and the fourth (chapter 6) was co-authored as well by Dr. Mary Senterman, who supervised the histological aspects of this work.

The investigations described in this thesis were designed and conducted by myself with the advice and consultation of Dr. Beverley Pearson Murphy. I formulated the protocols and conducted all the tissue culture experiments described. As well, I cultured and prepared all tissues for H/E staining. These staining techniques were carried out under the supervision of Dr. Mary Senterman by the pathology Department at the Montreal General Hospital. Except where noted, all assay determinations regarding concentrations of hormones given in this thesis were done by myself. The following is a brief outline of the chapters which constitute the thesis.

Chapter 1 reviews the important aspects of the human placenta ranging from its early development to physiological and endocrine functions. Human placental progesterone hormone

production has been emphasized and reviewed to date. A short review of tissue culture has also been included.

Chapter 2 describes in detail the experimental aspect of the work.

Chapter 3 reports the influence of certain androgens and a norandrogen on placental progesterone production in early placental explant cultures incubated with Ham's F-10 + 10% FBS. The compounds without effect in this regard were also mentioned. Comparative studies were also done with late placental samples. To observe the degree of aromatization of the progesterone stimulating steroids, estradiol production was also measured.

In Chapter 4, to assess the physiological significance of the progesterone stimulation obtained in Chapter 3, placental levels of steroids stimulating progesterone production were measured after chromatographic purification throughout gestation. 19-Nortestosterone levels were also measured before chromatography.

In Chapter 5 the mechanism of progesterone stimulation by androgens and a norandrogen was explored. The influence of progesterone stimulating steroids on the metabolism as well as on the biosynthesis were investigated in early placental

culture. The requirement of protein synthesis in association with progesterone stimulation is also reported.

Chapter 6 describes the improvement of basal progesterone production in the presence of human maternal serum (HMS). The viability of placental explants in the presence of HMS and FBS are also reported. Further, the nature of the stimulatory influence of HMS was also explored.

Chapter 7 closes the thesis with the summary and conclusions, and recommendations for future work.

Chapter 8 reports the contributions to knowledge made by this work.

CHAPTER I

HISTORICAL REVIEW OF THE HUMAN PLACENTA

1.1 Introduction

The placenta is a unique temporary organ through which the fetus communicates with the maternal environment to turn a fertilised ovum into a mature term baby. Since the mammalian ovum lacks the supply of nutrients for the developing embryos, the placenta is an indispensable element for the nutritive support of the fetus. Within its short life span the placenta develops as a highly specialized organ, with a wide spectrum of activities, acting as a multiple organ system of endocrine, respiratory, alimentary as well as excretory functions.

According to the different grades of contact between the mother and the fetus, human placenta is defined as haemochorial where maternal blood bathes the surface of the chorion. Thereby human placenta is distinguished from many other kinds of maternal and fetal contact that exists in different species (Pasqualini and Kincle, 1985).

1.2 Embryonic Development

The knowledge concerning implantation and development of human placenta has developed extensively; however finer details are not fully understood. The early embryological development of human placenta encompasses the time from

conception to the time of implantation. Immediately following fertilization of the ovum in the fallopian tube, the embryo undergoes cleavage during its passage toward the uterine cavity (Johnson and Everette, 1984). By three days after fertilization, the embryo has developed into a morula of 12-60 cells, which is then converted into a fluid-filled cavity, the blastocyst. This conceptus contains an inner cell mass of about 5 cells, the future embryo, and trophoblastic shell of about 53 cells that is destined to become the placenta. The initial development is confined to the outer ring, the trophoblastic shell. It is presumed that the trophoblastic shell at this time takes some nourishment from tubal secretions. Six days after fertilization (Martin, 1991), implantation begins with the attachment of the blastocyst to the uterine mucosa. Blastocyst invades the superficial decidua until it is completely embedded, having attained truly interstitial implantation. The structure actively concerned in excavating the uterine mucous membrane is the outer layer of blastocyst which proliferates to form the trophoblast, the placental component of the conceptus. As trophoblast proliferates circumferentially, it invades endometrial vessels to establish the primitive intervillous circulation. The primitive trophoblast quickly differentiates into an outer layer of multinucleated 'syncytiotrophoblast' and an inner mononucleated 'cytotrophoblast'. As gestation proceeds, cytotrophoblast cells become less numerous.

With the continuing trophoblastic invasion into the endometrium, lacunar spaces appear within the syncytiotrophoblast, which eventually become continuous with the maternal circulation in the intervillous spaces (Khong and Pearce, 1987). With continued development of trophoblastic trabeculae as a villous stem, from the proliferating cytotropholast the first appearance of primary chorionic villi occurs at the end of the 2nd week after fertilization. The secondary villi are formed by the development of mesenchymal cores inside the primary villi. With the differentiation of mesenchymal cores into blood capillaries, the secondary villi are now known as tertiary villi (Khong and Pearce, 1987). As pregnancy progresses, further differentiation of the placenta takes place (DeLia, 1990).

Decidua basalis, the maternal component of the placenta, is the transformed stromal part of the gestational endometrium underlying the implantation site, which supplies arterial blood to and venous blood from the lacunae in the intervillous spaces. The definitive placenta, chorion frondosum, is formed as a result of the continued rapid proliferation of the villi along the side of the chorion toward the decidua basalis with the regression of villi along the other side of the decidua. With the invasion of the decidua basalis by developing villi, 'placental septa' are formed dividing the placenta into lobules (Steven, 1983).

1.3 Gross Anatomy and Histology

As pregnancy advances, the placenta continues to grow approximating the size of the fetus in early second trimester (Flood and Hodgen, 1990). At birth the ratio of the weight of the placenta to that of the fetus is about one to six. The full term placenta is a disc-shaped organ having a diameter of about 29 cm with 2 cm thickness at the middle. Quantitative studies (Laga et al., 1973) of human placenta showed that term placenta weighs an average of 469 gm. Out of this, functional parenchyma was about 339 gm and nonparenchymal tissue was 130 gm. The chorionic villi (214 gm) consisted of 168 gm of peripheral and 46 gm of stem villi.

Histologically, placental parenchyma can be divided into villi and intervillous space. The microscopic appearance of placenta changes with the length of gestation (Teasdale, 1980). Mesodermal invasion in trophoblastic column identifies secondary villi. Tertiary villi composing the bulk of term placenta, contain blood vessels and a large amount of connective tissue. The inner layer of trophoblast (cytotrophoblast) is uninuclear and forms a single layer of cells, Langhans cells, which undergo mitosis and differentiate into the outer layer of trophoblast. Cytotrophoblasts have large nuclei and mitochondria with prominent nucleoli, the morphology common to embryonic tissue. With villous growth and capillary expansion, cytotrophoblast becomes sporadically discontinuous. It is at these areas where the syncytium may

abut the villous basement membrane directly. The syncytium is multinucleated and can not undergo mitosis. Its cytoplasm and nuclei are contributed constantly by the cytotrophoblast for the regeneration of syncytium (Richart, 1961; Galton, 1962). The syncytiotrophoblast is relatively thick in early placental development, thinning progressively throughout pregnancy. Langhans cells of cytotrophoblast get smaller and fewer after the first trimester and by term have disappeared. The syncytium is the most complex of placental cells containing a large variety of cytoplasmic structures. Electron microscopy reveals that the syncytium is filled with enzymes, innumerable transport vesicles, and has numerous lipid droplets. The core of the villous contains a varying number of stellate mesenchymal cells with small pyknotic nuclei. It also contains specialized fetal phagocytes namely 'Hofbauer cells' having large nuclei and vacuolated cytoplasm. At term the villous contains a large number of sinusoidally dilated fetal capillaries forming an important area of gas exchange (Kaufmann et al., 1979).

1.4 Immunological Aspect

The immunological functions of the placenta are important for the survival of the fetal allograft throughout gestation. Several mechanisms have been thought to prevent the fetus from immunological rejection (Sargent and Redman, 1987; DeLia, 1990; Faulk and McIntyre, 1983). One of the mechanisms that

seem to play a part is the nonspecific suppression of lymphocytes, the cell that would normally mediate the rejection of a graft. The activity of lymphocytes can be suppressed by a variety of potentially immunosuppressive substances produced by the human placenta. The substances claimed to be immunosuppressive are hCG, hPL, progesterone, cortisone and variety of other pregnancy specific proteins and glycoproteins. In comparison with other tissues, the placenta is minimally antigenic, expressing greatly reduced amount of MHC antigen. In addition, the placenta can react with anti HLA class I antibodies, internalize the complexes and destroy them. Another highly specific immunological function of the placenta is to supply the fetus with maternal antibodies - immunoglobulins.

1.5 Clinical Aspect

The placenta is usually attached to the fundal part of the uterus. The development of the normal placenta with its physiological function is an integral part of a successful pregnancy. Abnormalities of placental implantation can result in several clinical entities including early pregnancy failure (Hayashi, 1987). The essential role of the placenta is to maintain fetal well being through efficient exercise of the metabolic and respiratory functions of that organ. The progressive reduction of the placental function over an extended period of time results in the syndrome of intrauterine growth retardation with eventual fetal death.

1.6 Nutrient Transport and Gas Exchange

The placental transport mechanism plays a vital role in the development of the embryo and fetus. The placenta is a complex vascular organ adapted to optimize exchange of gases, nutrients and electrolytes between the maternal and fetal circulations. According to current concepts the exchange of substances between mother and fetus can occur by mechanisms ranging from passive diffusion to active transport and pinocytosis (Hill and Longo, 1980). Several factors can influence placental exchange, such as concentration differences, exchange area, the thickness and permeability of the placental barrier, molecular size, lipid and water solubilities, degree of ionization and molecular configuration of the transport materials.

From a functional point of view, the placental exchange is a phenomenon of concurrent exchange; oxygen and nutrients move from maternal to fetal blood through the placental membranes, while carbon dioxide and other fetal metabolites such as urea move in the opposite direction (Tropper and Petrie, 1987). The physiology of placental transfer has been reviewed by many investigators (e.g. Ahokas and Anderson, 1987; Faber and Thornburg, 1983).

Carbohydrate Transfer

A fetus requires a continuous influx of fuels for combustion and of building materials for the construction of

its body. The major metabolic fuel for the fetus obtained from its mother is glucose (Kalhan et al., 1979) a molecule of moderate size (MW = 180) which does not cross the placental barrier as rapidly as some smaller molecules. Sugar transport across the placenta takes place by means of a facilitated process as judged by the fact that the rate of transport does not increase indefinitely as the amount of substrate is increased but rather tends to reach saturating levels. In this process the glucose molecule is bound to a carrier molecule which is lipid-soluble, thus speeding the rate of transfer without involving energy expenditure. Placental glucose transfer is stereospecific, providing evidence of facilitated transport. Glucose, an aldohexose, is transported more rapidly than fructose, a ketohexose, and other sugars with similar molecular weights; D-xylose and D-glycose are transported more rapidly than their L-stereoisomers (Longo and Kleinzeller, 1970).

Protein and Amino Acid Transfer

Maternal protein transfer, although not nutritionally significant, is of great importance as it confers passive immunity to the developing fetus (Schlamowitz, 1977). The human fetus is born with significant titre of IgG (Gitlon and Gitlon, 1975) and receptors for IgG have been demonstrated on human placental membranes (Johnson and Brown, 1981). Although several hypotheses explain the transport of maternal immunogl-

obulins to the fetus, all involve binding to specific receptors on cell membrane (Faber and Thornburg, 1983).

Maternally derived amino acids serve as the major nitrogenous precursor for the synthesis of most of the fetal proteins. Fetal levels of amino acids are higher than those of the mother (Faber and Thornburg, 1983). An active transport process is required to transfer the individual amino acids against a concentration gradient. Naturally occurring amino acids i.e., the L-amino acids cross the placenta more rapidly than do the D-isomers which exchange at the rate expected on the basis of purely passive diffusion. There is competition of amino acid transport for the same carrier sites which become saturated at high concentrations. Neutral amino acids are preferentially transported by separate transport systems for certain amino acids (Lemons et al., 1979). Preferential transport systems for acidic and basic amino acids have not been defined yet.

Lipid Transfer

Fat is a significant source of energy for the fetus. Plasma albumin-bound free fatty acids (FFA) are potentially available for transfer across the placenta to the fetus for its fat synthesis. The rapid exchange of FFA suggests a possible mechanism of diffusion for the release of FFA from the protein complex into the lipophilic placental membrane. The more complex lipids are transferred as lipoproteins.

Vitamins, Minerals, Water and Fetal Waste Transfer

As cofactors or coenzymes for various metabolic pathways, vitamins and minerals are needed by the fetus. Fat-soluble vitamins readily cross the placenta probably by passive diffusion. The higher level of water soluble vitamins in the fetus than in the mother suggests active transport (Baker et al., 1981). Many ions such as chloride, sodium and potassium move readily across the placental barrier by diffusion. On the other hand, some ions seem to be actively transported against a concentration gradient. These include calcium, iron, iodine and phosphate, all of which have higher concentrations in fetal than in maternal circulation. Maternal-fetal water exchange results from complexly balanced osmotic and hydrostatic pressures.

The placenta is easily permeable to fetal waste such as urea and bilirubin, which cross to the maternal circulation by simple diffusion.

Gas Exchange

The respiratory function of the placenta is the transfer of an amount of oxygen from maternal into fetal blood adequate for the support and growth of the fetus; and the transfer of the CO_2 produced by fetal metabolism in the reverse direction. The transfer of respiratory gases across the placenta occurs by simple diffusion. According to Fick's law of diffusion, several factors such as concentration gradient, surface area

for exchange, and thickness of the placenta, influence the gas transport which is also facilitated by the high concentration of haemoglobin in the fetus.

Maternal blood releases oxygen to the fetal blood and at the same time accepts fetal metabolites which cause a fall in the pH of the maternal blood. This results in a shift of the maternal oxygen dissociation curve to the right (Bohr effect) which automatically increases the mass transfer of oxygen to the fetus by releasing oxygen from oxyhemoglobin. The transplacental movement of hydrogen ions also facilitates oxygen transfer. Transplacental CO_2 exchange follows the principle which regulate oxygen transfer. Transplacental CO_2 exchange is facilitated by the higher diffusion constant for CO_2 and by the simultaneous uptake of O_2, increasing the CO_2 combining power of blood (Haldane effect).

1.7 Endocrine Aspects

In the human, the placenta is a major endocrine gland which has been widely studied. A large number of steroidal and nonsteroidal hormones are synthesized by this complex and dynamic endocrine gland (Martin et al., 1991). Human beings have a villous haemochorial placenta in which trophoblast is in direct contact with maternal blood. The placenta can extract necessary precursors and intermediates from either fetal or maternal circulations. Many of the enzyme systems needed to synthesize steroid hormones from acetate are not

available in the placenta (Jaffe, 1983).

Placental nonsteroidal hormones are produced from the outer syncytiotrophoblast layer. The major protein and glycoprotein hormones produced by the placenta include human chorionic gonadotropin (hCG), human placental lactogen (hPL), human chorionic thyrotropin (hCT), human chorionic corticotrophin (hCC), beta-endorphin and pregnancy specific beta-1 glycoprotein (Flood and Hodgen, 1990). For clinical purposes only hCG and hPL have been extensively investigated.

1.7.1 Human Chorionic Gonadotropin (hCG)

The first placental glycoprotein hormone to be recognized, described in the literature (Ascheim and Zondek, 1927), was hCG with a molecular weight of 36-40 kDa (Swanithan and Bahl et al., 1970) having biological and immunological similarities to pituitary luteinizing hormone. Like other glycoproteins (LH, FSH, TSH), hCG consists of two subunits, alpha (α) and beta (β) (Bahl et al., 1972). Since the α subunit is common to other glycoproteins, the β-subunit is responsible for the specificity of hCG. The biological activity is only provided by the intact molecule of hCG.

The presence of hCG either in serum or urine is used as an accurate and sensitive determinant of human pregnancy. HCG was found to be detectable in serum as early as eight days after ovulation with a long half-life of 32-37 hours (Jaffe, 1983). Early in pregnancy the increase of hCG production is

logarithmic, reaching a peak (70-200 IU/mL) by 40-90 days gestation and followed by a sharp decline to about one-tenth of the peak value (20 IU/mL) by 14 weeks; it then remains fairly stable until the end of pregnancy (Marrs & Mishell, 1980).

The luteotropic function of hCG is well recognized; it maintains the production of progesterone needed for the support of early pregnancy. Little is known about the factors which regulate the huge production of hCG in pregnancy. Effects of various steroids, particularly progesterone, on hCG production have been documented by many authors, although the reports are not consistent (Wilson et al., 1984; Ahmed and Murphy, 1988).

1.7.2 Human Placental Lactogen (hPL)

HPL, an important metabolic hormone of pregnancy is one of the main proteins synthesized by the human placental syncytiotrophoplast (Sato, 1974). It is a single chain polypeptide with a molecular weight of 19-30 kDa. It has biological and immunological similarities to human growth hormones. Placental production of hPL increases steadily with placental weight as pregnancy develops (Martin et al., 1991). Although hPL is detected in serum (7-10 ng/mL) 20-40 days after fertilization (Josimovich, 1983), unlike hCG the usefulness of hPL for the detection of early human pregnancy is limited.

The growth-promoting activity of hPL appears to be mediated through its glucose-sparing effect, whereby the fetus is allowed to consume more glucose for its growth (Osathanondh & Tulchinsky, 1980). HPL has lactogenic activity in various bioassays and presumably contributes to preparing the mammary gland for lactation.

1.7.3 Androgens

In general C_{19} steroids are referred to as androgens on the basis of their androgenicity applied to some biological functions of the steroids. Due to the deficiency of C_{17-20} desmolase for side chain cleavage of C_{21} steroids, de novo synthesis of the principal androgens, dehydroepiandrosterone (DHA) and its conjugated form (DHAS), in the human placenta is not possible (Sobreville et al., 1964). During human pregnancy, placenta is supplied with the large amount of DHA and DHAS through maternal as well as fetal circulation (Simmer et al., 1966).

In peripheral plasma, the non-pregnant level of DHA (0.24-1.46 μg/100 mL) is similar to the pregnant level (0.37 - 0.68 μg/100 mL) (Gandy, 1971). During pregnancy (6-42 weeks), maternal plasma levels of unconjugated DHA do not vary significantly (7.33 ± 3.17 ng/mL) (Nieschlag et al., 1974). However, the concentrations of DHAS in non-pregnant serum (1-4 μg/mL (data from our laboratory) decrease with advancing gestation from 0.6-1.5 μg/mL at 6 and 26 wks to 0.4-0.8 μg/mL

at term respectively (Nieschlag et al., 1974; Buster et al., 1979).

Placental metabolism of DHAS consists primarily of hydrolysis to the free steroid and the conversion to Δ^4-3-ketosteroids. The presence of a very active steroid sulfatase system (Lamb et al., 1967) as well as 3β-hydroxysteroid dehydrogenase enzyme activity (Goldman, 1966) in placental preparations, are well established. With the aid of these enzymes, DHAS is converted into androstenedione more efficiently than the nonsulfated form (DHA) (Lamb et al., 1967). Testosterone is biosynthesized mainly from other fetal androgens, androstenediol sulfates (17β and 17α isomers) brought to the placenta. Due to the presence of placental 17β-hydroxysteroid dehydrogenase (Engel et al., 1974), the reduction of the 17-keto to 17β-hydroxy-4-ene C_{19} steroids takes place. Though a considerable amount of androstenedione is produced from the conversion of testosterone in the placenta, the reverse reaction is limited (Benagiano et al., 1967). Smith and Axelrod (1969) noted that human placenta as early as the fifth week of gestation was able to carry out different metabolic transformations of DHA.

In the above study, the <u>in vitro</u> metabolism of DHA included isomerization of the C_5 double bond and concomitant oxidation of the 3β-hydroxyl function, aromatization, reduction of the 17-keto function, 2-hydroxylation and 5α-reduction. Similarly the baboon placenta at term can metabolise

testosterone into different compounds : 4-androstene-3β,17β-diol, androstenedione, 19-hydroxyandrostenedione; 19-hydroxytestosterone, 19-aldotestosterone, estrone, estradiol, 2-hydroxyestrone and 2-hydroxyestradiol (Milewich and Axelrod, 1972).

1.7.4 Estrogens

Estrogens (estradiol, estrone, estriol) are 18-carbon steroids with one aromatic ring containing three double bonds between carbon atoms and one hydroxyl group on C_4. In pregnancy there is a progressive increase in the metabolic clearance of DHAS (Gant et al., 1971), indicating placental extraction of DHAS from fetal and maternal circulations. Placental biosynthesis of estrogens from androgen precursors, mainly from DHAS, has been known for a long time (Branchaud et al., 1983; Romano et al., 1986). The potent placental steroid sulphatase, 3β-HSD and aromatase (Osawa et al., 1987) metabolize androgen to estrogen through a number of intermediary products (Braselton et al., 1974). A-dione transformed from DHAS, is aromatized to estrone; the subsequent conversion of estrone to estradiol is mediated through 17β-HSD (Engel and Groman, 1974).

The production rates of estrone and estradiol do not differ significantly; both increase with the length of gestation (Pasqualini and Kincle, 1985). Estriol is synthesized in the placenta from the conversion of 16-hydroxyl-

ated C_{19} steroids originating in the fetal compartment. Estriol has much weaker biological effects compared to estradiol which has important metabolic functions including increases in body water and intravascular volume. Estriol excretion has been used to evaluate fetal health in obstetric complications (Ryan, 1980). The final conversion of C_{19} to C_{18} steroids involves 19-desmolation catalyzed by placental aromatase leading to the formation of estrogens. Non aromatizing 19-desmolase activity with the formation of 19-norandrogens has been reported recently (Osawa and Yarbough, 1983). Human placenta aromatizes 19-methylandrogens far more efficiently than 19-norandrogens (Silberzahn et al., 1988).

Although the physiological roles of estrogens during pregnancy are not well delineated, it is generally accepted that estrogens are required for maintenance of pregnancy and for growth of uterus and fetus. In general, estrogens are concerned with protein synthesis and cell proliferation within the endometrium, the myometrium, and the breast (Lauritzen and Klopper, 1983). Reduced maternal plasma and urinary estrogen concentrations are observed in certain fetal anomalies, severe intrauterine growth retardation and fetal death in utero (Pritchard et al., 1985).

1.7.5 Progesterone

Introduction

Progesterone (P4) can be considered to be the most primitive of reproductive steroid hormones. It is the principal steroid produced by the placenta during pregnancy. Progesterone is the only end product of short synthetic pathway, reaching higher plasma concentrations than the other steroids. The historical review of Little and Billiar (1983) noted the first purification of P4 from corpus luteum by Corner and Allen in 1929. Soon it was established as an essential hormone for the maintenance of pregnancy.

Chemical Form :

P4 is a C_{21} steroid containing the three six-membered carbon (phenanthrene) moiety attached to a cyclopentane ring. At carbons 10 and 13 of the basic structure, angular methyl groups are linked and at carbon 17, a side chain containing an ethyl group is attached. P4 (pregn-4-ene-3,20-dione) has a double bond between carbons 4 and 5 and ketone groups at carbons 3 and 20.

Source

P4 is synthesized in virtually all types of vertebrate steroidogenic tissues, e.g. ovaries, testes, adrenals and placenta. Recently Mitchel et al. (1987) reported the capacity of human decidua and fetal membrane to synthesize and metabolize P4. In primates, ovaries are essential only for a

brief period after fertilization. After about seven weeks (the luteo-placental shift) the placenta can produce enough P4 to maintain pregnancy (Csapo et al., 1973) up to the time of delivery. The observations of higher P4 concentration in the umbilical vein (37 μg/100 mL) than in the umbilical artery (14 μg/100 mL), the lack of change in urinary pregnanediol excretion in pregnant women after intra-uterine death, fetal removal or ligation of the umbilical cord, and the sharp drop of maternal plasma P4 level after the removal of the placenta - all confirmed the placenta as a major site of P4 synthesis and the lack of participation of the fetus in this respect (Pasqualini and Kincle, 1985).

Synthesis

Most mammalian cells can synthesize cholesterol from acetate. Available evidence indicates that placental metabolism of acetate to cholesterol is limited (Ryan, 1980). Labelled P4 was not retrieved from placenta perfused with radiolabelled acetate, although pregnenolone was efficiently converted to P4 (Sybulski and Venning, 1961). In 1945, Bloch administered deuterium-labelled cholesterol to a pregnant woman and showed radio-labelled pregnanediol in her urine. This was the first demonstration that maternal cholesterol was the physiological substrate for placental P4 production (Bloch, 1945). Using choriocarcinoma cells in culture, Simpson et al. (1978) showed the association of placental

cholesterol utilization with a specific protein - low density lipoprotein (LDL). Maternal cholesterol esterified and transported by LDL binds to high affinity receptors on trophoblast cells (Simpson et al., 1979), where it is internalized. The LDL cholesterol - ester complex is then fused with lysosomes and free cholesterol is liberated which is then utilized by the trophoblast in P4 synthesis via pregnenolone (Winkel et al., 1980; Simpson and MacDonald, 1981). The cholesterol side-chain cleavage enzyme 20α-hydroxylase, for the conversion of cholesterol to a 21-carbon steroid, pregnenolone - a rate limiting step - is present in the mitochondria of all steroidogenic tissues. Pregnenolone in the mitochondria is then converted into P4 in two steps. The first involves the NAD+-dependent oxidation of pregnenolone to preg-5-ene-3,20-dione and is catalyzed by a 3β-hydroxysteroid dehydrogenase (3β-HSD). The second involves the isomerization of pregn-5-ene-3,20-dione to give P4, catalyzed by the Δ^{4-5} isomerase enzyme system (Simpson and MacDonald, 1981; Ryan et al., 1966, Ryan, 1980).

Production Rate

During the early weeks of pregnancy, daily P4 production is approximately 55 mg and reaches 100 mg at mid-gestation. During the final months of gestation the overall production rate of P4 is about 210 mg/24 hours (Little and Billiar, 1983).

Progesterone is rapidly cleared from blood; its metabolic clearance rate (MCR) is approximately 2100 L in the menstrual cycle. The MCR of P4 does not vary during pregnancy, so that the differences in production rate vary directly with levels of P4 (Lin et al., 1972).

While animals such as the sheep exhibit dramatic decreases in circulating P4 immediately before the onset of labor (Csapo et al., 1971; Turnbull et al., 1974), such observations have not been made with any consistency in the human (Anderson et al., 1985; Hartikainen et al., 1981; Mather et al., 1980; Borditsky et al., 1978,).

In the circulation, progesterone is bound to plasma protein particularly corticosteroid-binding globulin (transcortin) (Diamond et al., 1969) with approximately same affinity as cortisol. P4 also binds to plasma albumin and orosomucoid to a lesser degree (Westphal, 1970). A specific progesterone-binding protein appears predominantly during pregnancy only in the guinea pig (Milgrom et al., 1973). Only a small percentage (4% - 5%) of progesterone in the circulation exists in an unbound state and does not change throughout pregnancy (Tulchinsky and Okada, 1975). In the rabbit uterine fluid, P4 is carried by a dimeric protein, uteroglobin (Savouret et al., 1984). As it is a lipophilic steroid, progesterone is taken up in large amounts by body fat (Little and Billiar, 1983).

Plasma and Tissue Concentrations

Much of the information about progesterone was initially derived from measuring the inactive urinary metabolites. The main metabolite, pregnanediol, is 40 mg/day in maternal urine (Gower and Cooke, 1983). Salivary P4 is thought to reflect the unbound fraction of plasma P4, the pattern of which is similar during the menstrual cycle (Luise et al, 1981). Using a direct RIA for P4, Bourque et. al (1986) reported the salivary level of about 100 pmol/L and plasma level of 1 nmol/L in the follicular phase. During the luteal phase, salivary level peaks (to 400 pmol/L) as does the plasma level (to 26 nmol/L).

During pregnancy, there is significant variation in plasma P4 concentrations even in the same patient. The cause of this fluctuation is not known. Urine or saliva steroid assays may serve to integrate moment to moment variations in plasma progesterone concentration providing better estimates of production or metabolism (Gray et al., 1987; Khan-Dawood et al., 1988). Maternal plasma P4 concentrations (25-75 ng/mL) typically fall from 4 to 8 weeks gestation as the corpus luteum demise proceeds. Thereafter, plasma P4 levels steadily rise as pregnancy advances from 30 ng/mL in early pregnancy, to 100-300 ng/mL at term (Abraham et al., 1972; Tulchinsky et al., 1972). The retroplacental blood P4 levels average 850 ng/mL at term (Tulchinsky and Okada, 1975).

The large difference between umbilical venous (720 ng/mL) and arterial values (440 ng/mL) indicates placental secretion into the fetal circulation and fetal uptake of P4. Although most of the P4 produced by the placenta is secreted into the uterine venous blood and only 10% goes to the fetus, the fetus is exposed to high concentrations of P4. Fetal serum levels are nearly seven times those seen in the maternal serum (Ryan, 1980). The high P4 level in umbilical cord at term is surprising since fetal corticosteroid- binding globulin is lower in cord blood than maternal plasma. The physiological significance of the high fetal plasma P4 level may be related to its important role in the regulation of fetal steroidogenesis where P4 serves as a substrate for the production of adrenal corticosteroid hormone (Tulchinsky and Okada, 1975). The placental concentration of P4 is about 10 times higher than that in fetal tissue (Runnebaum et al., 1975). Recently, Ahmed (1990) reported preliminary data which suggests that the placental concentration of P4 peaks early in gestation.

Metabolism

P4 is largely metabolized by dehydrogenations and reductive pathways in the placenta. The stereospecific reduction of C_{20}-ketone to 20α-dihydroprogesterone (20α-DHP) has been reported by many investigators (Kitchen et al., 1967; Purdy et al., 1964; Little et al., 1959).

In vitro studies failed to demonstrate any 20β-hydroxysteroid dehydrogenase activity in midterm and term placenta (Hart, 1966). In late human placenta, 20α-hydroxysteroid dehydrogenase enzyme activity was characterized recently (Rabe et al., 1982). The average concentration of 20α-DHP in placental tissue (461 ± 156 ng/gm) is only twice as high as in the fetal plasma (283 ± 109 ng/ml) (Runnebaum et al., 1975). The P4 metabolism to 20α-DHP (pmol/mg protein/hr) was found to be increased in association with parturition (835 ± 103 before labor to 11,604 ± 101 after labor, $P < 0.05$) (Diaz-Zagoya et al. 1979) suggesting its importance in the mechanism of initiation of labor. At midpregnancy, in situ perfusion experiments indicated that the interconversion of P4 and 20α-DHP in the placenta is strongly shifted in favour of P4, converting large amounts of 20α-DHP into P4, but only very small amount of P4 into 20α-DHP (Zander, 1964; Palmer et al., 1966). In the placenta the concentration ratio of 20α-DHP/P4 (1:10) is less than that in the fetus, where the high conversion of P4 to 20α-DHP is presumably a protective mechanism against the biological activity of the hormone itself.

The placenta is also provided with 20α-DHP produced in the fetal compartment (Zander, 1964; Palmer et al., 1966). Although the physiological importance of the P4 metabolite (20α-DHP) in the human has not been established, Wilcox and Wiest (1960) reported that the bioactivity of 20α-DHP was

about one-sixth that of P4 in terms of deciduomata formation in the rat. In the human placenta, P4 is also metabolized to other compounds such as 6α- and 6β-hydroxyprogesterone (Tabei and Troen, 1975), 6-oxoprogesterone (Hagopian et al., 1956) and 16α-hydroxyprogesterone (Jungmann et al., 1967). The metabolites 5α-pregnane-3,20 dione and 3β-hydroxy-5α-pregnane-20-one are also identified in the human placenta (Milewich et al., 1979). The production rate of 5α-pregnane-3-20 dione is in the range of 0.5 mg/human placenta/day (Milewich et al., 1979).

P4 metabolism into C_{19} steroids by side chain cleavage has not been confirmed in the human placenta: a report of <u>in vitro</u> conversion of P4 into androstenedione and testosterone (Jungmann et al., 1967; Warren et al., 1964) in placental preparations was not confirmed by many investigators (Kitchen et al., 1967; Palmer et al., 1966; Sobreville, 1964), who failed to find C_{19} steroids from P4 following incubation or perfusion of midterm and term placentas indicating C_{17-20} desmolase is not active in the human placenta. When labelled P4 was injected into the maternal circulation, many steroids resulted, including pregnanolone, 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnane-20-one (Eriksson and Gustfsson, 1970).

Maternal liver and kidney are sites of further metabolism; circulatory P4 is cleared from plasma with a short half-life of 10-15 minutes. All metabolites are glucuronide- or sulphate-conjugated. The estimation of the major metabo-

lite of plasma progesterone (pregnanediol glucuronide) is truly a placental function test since placental P4 secretion continues unchanged even after fetal death (Coyle et al., 1962). Recent study of P4 metabolism in plasma suggested that 50% of the P4 metabolism in late gestation is accounted for by the formation of sulfated steroids with a 3α-hydroxy-5α configuration (Anderson et al., 1990).

Physiological effects of P4 :

P4 is known to play a fundamental role in the establishment and the maintenance of pregnancy. Many biological responses other than those related to pregnancy are also elicited by P4 action on different organs including the endometrium, myometrium, kidney, breast, brain and liver (Savouret et al., 1990; Rothchild, 1983; Pritchard et al., 1985; Little and Billiar, 1983; Fuchs and Fuchs, 1984; Lipset, 1986).

The physiology of P4 action in human pregnancy is poorly understood. In the primates, P4 is usually considered to have two principal effects on the uterus. The first is the ability of P4 to convert the endometrium from a proliferative to a secretory type which is essential for successful implantation of the fertilized ovum. Decidualization of endometrium induced by P4 is associated with changes in mucopolysaccharides and in the connective tissue components of the stroma. Decidual cells having marked biosynthetic ability synthesize

several endometrial proteins such as prolactin and inhibitors of plasminogen activators (Joshi, 1983). In the rabbit, a direct effect of P4 is to cause the endometrial stimulation of synthesis of a specific protein - uteroglobin - representing 40 - 60% of the total protein in the uterine fluid (Savouret et al., 1984). Although the biological action of uteroglobin is not clear, it binds to P4 giving high levels of P4 in the uterine fluid.

Other biochemical changes induced by P4 include histamine release and a decrease in the number of mast cells, leading to vasodilatation and edema. The morphological changes in the microvilli along with electrochemical changes stimulated by P4 facilitate the implantation of the embryo.

The most widely discussed effect of P4 in pregnancy is its ability to inhibit uterine muscular contractions. In sheep P4 is known to block myometrial contractility by increasing calcium binding to the sarcoplasmic reticulum and myometrial cell membrane (Csapo, 1977). Although P4 maintenance of uterine quiescence does not appear to be firmly established, pharmacologic treatment with P4 and synthetic progestational agents has proven effective in preventing premature labor (Erny et al., 1986).

The inhibitory influences of P4 on myometrial excitability is thought to be related to the gap junctions, the formation of which is suppressed by P4 (Garfield, 1982). Since P4 maintains lysosomal membrane stability, it inhibits

the release of phospholipase A_2 from lysosomes to hydrolyze arachidonic acid esters for the synthesis of prostaglandin F2α and PGE2, known to affect myometrial contractility (Little and Billiar, 1983).

Reductions in progesterone tend to favour all factors that stimulate uterine contractility. Animal studies indicate that progesterone may inhibit the onset of labor in a number of ways including inhibition of the effect of oxytocin, decreasing prostaglandins F2α synthesis, reducing myometrial sensitivity to prostaglandins F2α, decreasing α-adrenergic effects. Although a decrease in progesterone hormone probably triggers the onset of labor, the precise role of progesterone in parturition in human pregnancy is still controversial (Fuchs and Fuchs, 1984).

In conjunction with estrogen, P4 exerts a direct effect on the glandular tissue of the breast, making it suitable for lactation. It is presumed that progesterone acts as a competitive inhibitor of aldosterone in the distal renal tubules resulting in sodium loss. This effect may be compensated by stimulation of the renin-angiotensin system and increased secretion of aldosterone. Progesterone increases the pancreatic islet response to a glucose stimulus in that a greater and more sustained rate of insulin release is observed (Gyton, 1986).

Progesterone is considered to be a native immunosuppressive agent acting to prevent immunological

rejection of the fetus by the mother. P4 has demonstrable immunosuppressive properties such as local anti-inflammatory and graft-sparing effects, inhibition of human and murine lymphocyte activation and the generation of killer T-lymphocytes (Flood and Hodgen, 1990). P4 is known to inhibit phytohemagglutinin (PHA) stimulated activation of human T lymphocytes (Clements et al., 1979; Stites and Siiteri, 1983). Since progesterone interferes with T-lymphocytes cell mediated tissue rejection, it confers immunological privilege to the implanted products of conception. High intervillous concentrations of progesterone may play a major role in blocking rejection of fetal antigens (Pritchard et al., 1985).

Molecular Action of P4

Although P4 is known to play a fundamental role in the regulation of the female reproductive process, the mechanism of P4 action is poorly understood. Comprehensive studies with the chick oviduct system have demonstrated the importance of a specific receptor-hormone complex in active intracellular sites. The molecular effects of P4 through binding to the specific intracellular protein-receptor, has been reviewed recently (Savouret et al., 1990; Clark, 1988; Milgrom, 1981).

According to the generally accepted model of steroid hormone action, P4 binds strongly to receptor protein located in target cells. The hormone receptor complex, in turn, acquires high affinity for a limited number of acceptor sites

composed of chromosomal proteins and/or DNA. Receptor-acceptor binding eventually culminates in transcriptional activity of specific genes. P4 action in rat placenta fits within this general framework (Ogle, 1986). Recently progesterone receptor (PR) has been identified in the human placenta at term (Rivera and Cano, 1989). Human cancer study also showed PRs in breast, kidney, prostate and in endometrial carcinoma.

The PR is best characterized for chick oviduct. It is a dimer having two subunits A and B, with molecular weights of 79 kDa and 110 kDa. P4 binds to the complete receptor with high affinity (K_D = 1 nM); however the resultant complex dissociates rapidly. For the purification of PR, a monoclonal antibody against human uterine PR has been obtained recently. A complete amino acid sequence of human PR cDNAs suggested a common (with other steroid hormone receptors) cysteine-rich central zone associated with the DNA-binding function of receptors. The gene for the human PR has been localized to chromosome 11q22-q23 (Rosseau-Merk et al., 1987).

Estrogen induction of PR formation in the target cell provides a priming effect for subsequent progestational response in the target tissues. The concentration of PR is down-regulated by P4 hormone itself and up-regulated by epidermal growth factor (EGF) and prolactin along with estradiol.

Although the participation of PR-nuclear interactions in

the mechanism of progestin action has been suggested in the uterus, other mechanisms may be involved (Little and Billiar, 1983). For instance, P4 induction of meiosis in the frog oocyte was thought to be mediated by direct modulation of membrane activities.

The inhibitory effect of P4 on membrane-bound adenylate cyclase caused a decreased level of cAMP without involving the nuclear PR. P4 was found to modulate the concentration or affinity of membrane receptors for catecholamines as well as for oxytocic compound (Finidori et al., 1982). As an antiprolactin, P4 suppresses the formation of milk protein by inhibiting its transcription and mRNA accumulation. A direct inhibitory effect of P4 on prostaglandins biosyntheses was mediated through stabilization of lysosomal membrane and perhaps through some permeability effect (Savouret et al., 1990)

Regulation of P4 Production in Placenta

The important roles of placental progesterone in maintaining human pregnancy is undisputed. However, the regulation of placental P4 production has not yet been elucidated. Neither hCG nor the fetus seems to exert any significant control over placental P4 production (Menon and Jaffe, 1973), suggesting a local hormonal regulation. Steroid modulation of P4 synthesis has been reported by many investigators (see Chapters III and V), however, the reports are not consistent.

Moreover, most of the previous studies were focused on mid-term and term placenta rather than on early tissue (Ahmed, 1990). Little work has been done yet regarding placental P4 regulation during the early part of gestation when there is a transition from luteal to placental dominance of P4 production.

1.8 TISSUE CULTURE

Because of the complexities of whole animal experimentation, the concept of *in vitro* tissue culture system has evolved along two different lines for the continuation of *in vivo* physiological functions. One approach is organ culture, where tissues, organ primordia or the whole or parts of an organ are maintained *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function (Federoff, 1967, 1975). The second approach is monolayer or cell culture where tissues are dispersed into cellular units before culturing *in vitro* to look at the biology of individual cells. According to Stromberg (1980), the explant culture is distinguished from organ culture only by the smaller size of the initial segment of the tissue used. The explant consists of an organised collection of cells, held together by an intracellular matrix, similar to that which exists *in vivo*. An essential difference between an explant culture and a cell culture is the absence of cell interaction in dispersed cell cultures, which is probably of functional significance. It is now recognized that associated cells exchange signals via junctional communications and via paracrine hormones and surface information exchange (Lasnitzki and Mizuno, 1979).

The importance of cells being in association was reported earlier: alveolar cells of the lung will only synthesize and

release surfactant in response to hormonal stimulation of adjacent fibroblasts (Post et al., 1984). Epithelium differentiates in response to matrix constituents, often determined jointly by the epithelium on one side and connective tissue stroma on the other (Hodges, 1969), as may be the case with the interaction between epidermis and dermis in vitro (Bohnert et al., 1986). The above studies suggest that to learn about the integrated function of the whole organism an organ culture model is required.

Another limitation of cell cultures is the profound alterations which they frequently undergo during the course of serial propagation (Fell, 1976; Hodges, 1976). Enzymes, chelating agents or mechanical agitation are widely used for tissue dissociation as cell isolation procedures. Most of these methods cause some degree of trauma to the cells and a variety of cellular alterations have been recorded following trypsin and other enzyme treatment of tissues (Waymouth, 1974). The adverse effect of tissue dissociation, resulting in the decline of production of hCG by the trophoblast, increases with the increasing period of trypsinisation (Loke, 1983). In addition, the preparation of cell culture is labor intensive.

With all these limitations of tissue dissociation, it is the 'structural integrity' which is the main reason for adopting explant culture in this study as an in vitro technique, in preference to cell culture. A major deficiency of

explant culture is the absence of a vascular system, limiting the size of the explant by diffusion. To avoid this problem, placental cotyledons have been perfused recently (Bloxam et al., 1986; Brandes et al., 1983; Contractor et al., 1983). However, owing to the complex apparatus requirement and technical difficulties of using it, the perfusion technique has not been employed in the present study.

When cells are cultured as a solid mass of tissue, the gaseous diffusion and the exchange of nutrients and metabolites become limiting; central necrosis is often apparent (Hodges, 1976). To alleviate this problem, organ cultures are usually placed at the interface between the liquid and gaseous phases to facilitate gas exchange while retaining access to nutrients. Several techniques have been described for gas-liquid interface culture (Bjerkvig et al., 1986; Nicosia et al., 1983), but one which has become popular is the grid technique of Trowell (1954, 1959). This technique has been widely used in many studies (e.g. Ahmed and Murphy, 1988; Abramovitz et al., 1982; Huot et al., 1979).

The object of organ culture is the maintenance of structural and functional integrity of the explanted tissue. Therefore, to minimise the possible loss of tissue organization, traumatic dissection procedures have been avoided by using sharp dissecting instruments: fine scissors, forceps and surgical blades. Based on estimations of the rate of oxygen diffusion through a given tissue, the size of the tissue

explant is predicted (Trowell, 1961). As in this study, the chorionic villi are usually minced into small fragments of approximately 1-3 mm^3. However, relatively large explants of 1 cm^3 have been cultured successfully by others (Stromberg et al., 1978; Huot et al., 1979).

Tissue age may influence organ culture (LeDouran, 1970). In this study as well as in others (Ahmed and Murphy, 1988; Huot et al., 1979), placental explants were obtained from both early and term gestations (see Chapters 3, 5 and 6).

According to Biggers (1965), tissues removed from their original environment for <u>in vitro</u> culture, undergo three phases : the initial shock phase, the middle stable phase and final hydration phase. In accord with the above concept, the experiments in this study were carried out on day two after an initial equilibration of 24 hours unless otherwise indicated.

Tissues, in general, are cultured at temperatures within the range of the body temperature of the species from which they are derived (Monnickendam and Balls, 1973). In the present study, human placental cultures were incubated at the standard body temperature of 37^{0}C. Even the smaller pieces of tissue tend to get necrotic when completely immersed in culture medium (Hodges, 1976). Therefore, for the maintenance of viability, tissue should be in contact with the gas phase. In the present study, a gaseous environment of 5% CO_2 in air was generally employed. However, a wide range of oxygen concentrations have been used satisfactorily in placental

organ culture indicating unusual tolerance to changes in oxygen tension (Loke, 1983). According to the usual concentration in the alveolar spaces of the lung, 5% CO_2 is preferred for organ culture. In addition, each medium is formulated with a bicarbonate buffer system. To achieve the correct pH, 5%-8% CO_2 is necessary along with recommended bicarbonate concentration (Hodges, 1976) (see appendix 1)

1.9 Purpose of the present study

The function of the human placenta is of interest for both scientific and practical reasons. Unfortunately *in vivo* study is very difficult because such studies usually require the use of radioisotopes or repeated sampling of fetal blood; both requirements carry unacceptable risks. One must therefore try to learn about human placental function by choosing an *in vitro* human system or appropriate animal model. Since it is potentially dangerous to extrapolate results from the animal to the human situation, I prefer to use an *in vitro* system.

The importance of the luteo-placental shift in progesterone production for the support of human pregnancy is generally accepted. While the pathway by which placental progesterone is synthesized is understood, the factor(s) responsible for the regulation of progesterone production have not yet been defined, particularly during early gestation.

To investigate the factor(s) responsible for the regulation of placental progesterone production at the time of luteo-placental shift, the present study aimed at the following specific objectives :

1. To investigate the effects of various steroids and peptides on placental progesterone production in culture.

2. To measure the placental concentrations of hormones having influences on progesterone production in culture.

3. To explore the mechanism of progesterone stimulation.

4. To improve the culture conditions for *in vitro* hormone production.

The availability of abortion material from first trimester pregnancies made this study feasible. Placental samples at other gestational ages have also been studied for comparison.

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CHAPTER II

EXPERIMENTAL

2.1 Materials

Placental Samples:

Tissue samples were obtained at socio-medical abortion, hysterotomy or at delivery from normal healthy women attending local hospitals. According to the medical records, none of them were on hormonal contraceptives or on any other drugs. Gestational age of placenta was estimated by the date of the last menstrual period (LMP) or by the measurement of foot length (FL) of the fetus (Munsick, 1984). There was good agrement as to the gestational age assessed by LMP and FL (personal verification).

Tissue Culture Equipment:

Edgegard laminar flow hood: Baker Co.(Biddford, Maine)

Incubator: National, Portland, Oregon.

Glass bottles (100-500 mL): Grand Island Biological Co.(GIBCO)

Pasteur pipettes, scissors, forceps: Fisher Scientific Co., Montreal.

Millipore filters (0.22 μ): Millipore Co., Bedford, Mass.

Plastic culture dishes (60 x 15 mm): Falcon Plastics, CA.

Stainless steel grids (2 x 1.5 cm, cut from 40 mesh stainless steel): Johnson Wire Works, Montreal.

Surgical blades (no. 10): Paragon Razor Co., Sheffield, England.

Ham's F-10 nutrient mixture, fetal bovine serum : GIBCO

Human maternal serum: local pregnancy termination unit.

Solvents, Reagents, Chemicals:

Sodium monophosphate ($NaH_2PO_4.2H_2O$), sodium diphosphate ($Na_2HPO_4.7H_2O$), scintillation fluid (Optiphase), and commercial detergent (Sparkleen): Fisher Scientific. Co. Montreal.

Ethanol (95% and 99%): local pharmacy

Gelatin: local grocery

Dextran (G 201): Baker Chemical Co. Pittsburg, New Jersey.

Equipment and reagents for hormone assay:

Automatic horizontal shaker: Eberbach Corporation, Michigan.

Refrigerated centrifuge (IEC CENTRA-7R): Fisher Scientific Co., Montreal.

Assay tubes : Fisher Scientific Co., Montreal.

Pregnant guinea pig serum (50-55 days gestation): Animal Resource Centre, McIntyre Medical Science Building, McGill University.

Progesterone antiserum (R4): Raised in our laboratory against progesterone -6-succinate.

Progesterone antiserum (P11-192): Raised against progesterone-11-succinate. Endocrine Science, Tarzana, CA (courtesy Dr. Charlotte Branchaud, Montreal Children's Hospital)

Antisera for estrone and estradiol: Gift from Dr. Hamish Robertson (Ottawa, Ontario).

Counting of radioactivity:

All tritiated radioactivity: LKB-Wallac Rack-Beta Liquid Scintillation counter (LSC), Turku, Finland.

All iodinated radioactivity: LKB-Wallac (1272 CLINIGAMMA) automatic gamma counter, Turku, Finland.

2.2 Tissue Culture

2.2.1 Aseptic Technique

During the preparation of cultures, the living tissue is exposed to many potential contaminants which could irreversibly damage the cells. Therefore, aseptic technique is a basic requirement of in vitro culture. Particular care was taken to clean and sterilize each item before use in culture. Bottles or sample vials containing radioactivity and culture dishes were discarded. Immediately after use, other items of glassware were rinsed under tap water to prevent drying of material on them, thereby facilitating subsequent washing with commercial detergent in a sonic bath filled with hot water. After rinsing with tap followed by distilled water, the washed items were air or oven dried.

Prewashed bottles and other culture equipment (Pasteur pipettes, sample containers, scissors, forceps, etc.) were sterilized either by gas or steam autoclave at a temperature of 121^0C and at a pressure of 15 lbs/inch^2 for 20 minutes. Disposable culture dishes were received in sterile packages, all other culture equipment used was sterile.

To keep the working place free of airborne microorganisms, all preparations for culture were done under the laminar flow hood. Before and after using the hood, it was cleaned three times with 95% ethanol.

2.2.2 Antibiotics for Culture Medium

The following antibiotics were used:

Amphotericin B (50 mg/vial) - ten mL sterile water was added to the vial which was stored at 4^0C and used within ten days of preparation. To each 500 mL culture medium, 0.5 mL of prepared antibiotic was added after filtration of medium to have a final concentration of 5μg/mL.

Penicillin G (1 million IU/mL) - six mL sterile saline was added to the vial which was stored at 4^0C and used within two weeks of reconstitution. To allow the antibiotic to dissolve, it was added to the culture medium the day before use. For a final concentration of 200 IU/mL, 0.5 mL antibiotic was added to the 500 mL culture medium.

Gentamycin sulfate (80 mg/vial) - 0.5 mL was added to 500 mL medium to have a concentration of 40 μg/mL. It was used before the expiry date on the vial.

2.2.3 Tissue Culture Medium

Every set of experiments was done with a particular batch of freshly prepared medium. The powdered nutrient mixture, Ham's F-10, is formulated according to the basic nutritional

requirement of the living tissue (Gibco catalogue 1989/1990)(-see appendix 1). Reconstitution of the powdered medium was done following the guidelines written on the top of each package. The contents of package are poured in a clean container to which has been added an amount of distilled water 5% less than the required volume. With gentle stirring, the powder dissolved uniformly at room temperature. For a litre of medium, 1.2 gm of $NaHCO_3$ was measured on an analytical balance and added to the dissolved medium. Then, the volume was made up to exactly one litre with distilled water. Since filtration increases the pH by 0.1-0.3 units, before membrane filtration, the pH of the medium was adjusted to 7.0-7.2 with 0.1 N HCL to get a final working pH of 7.4 after filtration. Medium was sterilized by a membrane filter of 0.22 μ using vacuum suction. After filter sterilization, the medium was collected in a sterile bottle. Fetal bovine serum (FBS) was added at a concentration of 10%. Antibiotics were added to the medium before storing at 4^0C for 3-4 weeks or until the medium looked cloudy. All the cultures were done with medium composed of Ham's F-10 + FBS 10%, unless otherwise indicated.

2.2.4 Explant Culture

Fresh placental samples, after thorough rinsing with sterile saline, were collected in a sterile specimen container. Placental villi were identified, dissected with scissors, and minced into small pieces of about 1 mm^3 size.

Ten such pieces were carefully placed on two supporting grids (5 / grid) in a disposable sterile culture dish containing 3 mL of the culture medium. Cultures were incubated at 37^0C, in a humidified environment of 5% CO_2 in air. The culture medium was changed daily unless mentioned otherwise. The widely accepted chemically defined medium, Ham's F-10 (Ham, 1974) supplemented with FBS 10% has been used in this study unless otherwise indicated (see Chapter 6). The amount of tissue medium was adjusted to 3 mL per dish so that the fluid medium would just reach the supporting 'grids'. Therefore, necrosis from total immersion of explants in the culture medium was avoided. For convenience, medium was changed every 24 hours (unless otherwise indicated), allowing sufficient time for the expression of the functional capacity of the placenta. Each experiment was set up from the same placenta using the same batch of freshly prepared culture medium. The contamination of cultures was prevented by careful maintenance of aseptic technique during the culture period.

Most of the experiments were carried out within one week of culture during which placental explants cultured in Ham's F-10 ± FBS 10% were viable from the biochemical as well as the histological point of view (see chapter 6).

Most organ cultures do not grow or, if they do, proliferation is limited to the outer cell layers (Abramovitz et al., 1982). In the present study no visible outgrowth of explants was noticed under the light microscope over the usual

short experimental period of one week or long culture period of 30 days.

Although it was difficult to isolate villous tissue from the early abortion material, careful explantation avoided the contamination of any explant with tissues other than placental villi. This was confirmed histologically (see Chapter 6).

Though there was variation between individual placental samples, the coefficient of variation of P4 production calculated from three quadruplicate cultures from three different samples was minimal (< 10%) (Table 2.1) indicating adequate precision of explant preparation in the present study.

Table 2.1. Precision of explant preparation

Sample	Observation (n)	P4 (ng/10mg/d-ish) mean± SD	Coefficient of variation (%)
I	4	38.5±2.2	5.6
II	4	58.0±4.0	7.0
III	4	26.5±2.7	9.2

2.3 Extraction of Sample Medium and Placental Tissue

To determine the recovery by extraction of [^{3}H] hormones into the commonly used organic solvents in the laboratory, a small amount of radioactivity (500 - 2000 cpm) was evaporated in the tube to which a suitable amount of sample medium or homogenised tissue in saline was added. Each extraction tube was vortexed for 1 min after adding 4 to 5 volumes of organic solvent. To separate the organic phase from the aqueous one, it was then centrifuged for 5 - 10 minutes at 2000 r.p.m. Using a pasteur pipette, the organic phase was carefully removed from the top (from the bottom in case of methylene chloride) leaving behind a thin layer, to avoid the contamination of the extract with a part of the aqueous phase.

After 2 to 3 extractions, the combined extracts were evaporated to dryness. The radioactivity of aliquot from the redissolved extract in ethanol, was counted for 5 minutes and compared with the radioactivity initially added. The per cent recovery was calculated as the recovered/initial radioactivity X 100. Since extraction recoveries for the hormones (P4, E_2, A-dione and 19-NT) were maximal (95% - 100%) in diethyl ether all extractions were done with diethyl ether.

2.4 Column Chromatography

2.4.1 General Technique of Column Chromatography

Sephadex is a bead-formed, cross-linked dextran gel which swells in water and aqueous salt solutions. By varying

the degree of cross-linking, gels of different porosities and with different fractionation ranges are obtained. Sephadex LH types are derivatives of Sephadex with wide applicability in the fractionation of lipids, steroids, fatty acids, hormones, vitamins and other smaller molecules. Sephadex LH-20 is prepared from Sephadex G-25 by hydroxypropylation. Separations are achieved by gel filtration according to the size of molecules. Such separations also involve adsorption partition, ion exclusion, retardation and other unknown mechanisms (Information booklet, Pharmacia Fine Chemicals, 1984).

The primary purpose of chromatography is to achieve the separation of closely similar substances. Sephadex LH-20 column chromatography has been established in our laboratory as an effective separation technique for different steroids such as androgens, estrogens, progestins, and corticoids (Murphy, 1971). Steroids can be purified by other methods such as thin layer or paper chromatography. However, the blank values of these techniques in competitive binding assays are usually higher, so that column chromatography was preferred. The high recovery and good reproducibility with low blank values (Murphy, 1971) make the system of column chromatography suitable for the present study.

Column chromatography requires, in principle, only a simple apparatus and easy procedures. The dry Sephadex was soaked for 3-4 hours in the solvent system to be used for elution (heptane : methylene chloride : ethanol - 50 : 50 :

1). As columns, 4 - 5 glass burettes (25 mL long and 0.9 cm internal diameter) were employed. A small piece of glass wool, barely enough to cover the mouth of the opening at the bottom, was wrapped at the end of a glass rod and placed at the bottom of the tube. By keeping the stopcocks open, Sephadex slurry was transferred into the column in small aliquots. As the solvent drained, the Sephadex settled. A uniform air-free column was packed by repeating the procedure of adding Sephadex slurry and letting the solvent drain off until the height of the Sephadex bed was 39 cm . The solvent was allowed to drain off until it just covered the Sephadex bed. To have a column free of gas bubbles, pores and cracks, slight pressure was applied occasionally with a glass rod. After packing the column, it was covered with a small piece glass wool in order to prevent the Sephadex bed from rising. Before running the columns, they were left for a day to settle and equilibrate with the elution solvent to be used. For running the samples in 0.1 mL solvent, the glass wool was removed from the top of each column. Before running the sample, several fractions were collected and checked for the blank value. After introduction of samples, fractions (1-5 mL) were collected manually at a flow rate of about 1 mL/5 minutes. To speed up the procedure, slight pressure was applied by means of a syringe attached to the top of each column.

Depending upon the respective reference steroids, certain

volumes of fractions were collected. All fractions were evaporated and redissolved in ethanol. Aliquots were counted to locate the radioactivity and to calculate recoveries. Suitable aliquots were also assayed to quantitate the various hormones. For all the hormones, the separations were consistent from all the columns. After finishing collection from one experiment, the columns were washed with 100 - 200 mL column solvent and were then ready to be used again. If this washing was insufficient to remove all the added material, the columns were dismantled and the Sephadex was washed repeatedly with ethanol and then allowed to dry in air before re-use.

2.4.2 Counting of Radioactivity

Radioactivity was determined after adding 2 mL scintillation fluid, Optiphase, to each dry sample or to each of those in 0.5 mL aqueous buffer (competitive binding assays) in a 4 mL plastic vial. For thorough mixing, all counting vials containing samples and Optiphase, were shaken well before placing into the counter. The counting of assay samples was continued for at least 10 minutes or until 10,000 counts were accumulated, the latter giving a counting error of ± 1%.

2.5 Hormone Analysis

2.5.1 Buffer Solutions and Gelatin Water

Phosphate (PO_4) buffer solutions are used routinely in our laboratory for protein-binding hormone analyses. The use of phosphate buffer in the hormone assay system was also reported earlier (Knobil, 1970).

Phosphate buffer for progesterone (0.075 M, pH 7.5) was prepared by dissolving 0.2 gm sodium monophosphate, 0.875 gm sodium diphosphate, 0.5 gm gelatin and 1.0 gm sodium azide in 1 litre of distilled water.

Buffer A (pH 6.8) was prepared by dissolving 5.38 gm sodium monophosphate, 16.35 gm sodium diphosphate, 9.0 gm sodium chloride, 1.0 gm sodium azide and 0.5 gm gelatin in 1 litre distilled water.

Buffer B (pH 7) was prepared by dissolving 5.38 gm sodium

monophosphate, and 16.35 gm sodium diphosphate in one litre of distilled water.

Phosphate buffer for androgen (0.2 M, pH 6) was prepared by adding 960 mL of 0.2 M sodium monophosphate (27.6 gm in 1 litre distilled water) with 140 mL of 0.2 M sodium diphosphate buffer (13.4 gm in 250 mL distilled water).

SHBG phosphate-gelatin buffer (pH 6) was prepared by adding 0.5 gm/mL gelatin to phosphate buffer for androgen.

The importance of gelatin in protein binding assay was reported from our laboratory (Murphy, 1974). The stability of one protein was found to be enhanced by the presence of another (Leyendecker et al. 1972). Gelatin was found to decrease the adhesion of the dilute binding protein (used in the assay) to glass. Therefore, in the presence of gelatin (0.5 gm/L) the binding of hormones with protein was found to be increased (Murphy, 1974).

2.5.2 Dextran Coated Charcoal Solution (DCC)

In our assays (except for androstenedione and β-hCG assays), a Dextran coated charcoal solution (DCC) was used to separate the bound fractions from the free fractions in protein binding reactions.

To prepare a DCC stock solution, 2.5 gm of Norit A charcoal and 250 mg of Dextran were added to 100 mL of distilled water. The working charcoal solution was prepared by diluting the stock solution 1:20 with the phosphate buffer B. During the period of dispensing, the charcoal solution was kept in a uniform suspension by a magnetic stirrer.

2.5.3 Steroid solutions

Unlabelled crystalline steroid standards used in this study were stored in a desiccator. Before preparing stock solutions, steroids were weighed carefully on the analytical balance. In general 10 mg steroid was dissolved in 10 mL of ethanol to prepare a stock solutions of 1 mg/mL. From the stock solutions, various dilutions were prepared. All the steroid solutions were stored at -10^{0}C. In all experiments, the required amounts of steroid solutions were pipetted and the ethanol was evaporated under air.

The tritium-labelled steroids used in this study were diluted with redistilled ethanol to a concentration of 10- 50 μCi/mL and stored at -10^{0}C. Occasionally the radiochemical purity of the radioactive steroids was checked by Sephadex LH-

20 column chromatography. In all the studies, the purity of radiolabelled steroids was more than 98%.

2.5.4 Protein Binding Assay

Two kinds of competitive protein-binding (CPB) (Murphy, 1964) assay were used in the present study namely, radiotransinassay (**RTA**) (Murphy, 1967) and radioimmunoassay (**RIA**) (Yalow and Berson, 1960). The only difference between RIA and RTA is in the nature of the binding protein. RTA employs hormone- specific binding proteins that exist normally in the body fluids and/or tissues; while RIA employs antibodies - specific binding proteins developed in a host animal in response to an immunological challenge by the compound of interest (antigen). Both naturally occurring and immuno types of stereospecific proteins have been successfully applied to hormone analysis.

In CPB a compound is measured according to its ability to compete for a limited number of binding sites with a tracer possessing similar binding characteristics (Murphy, 1964 and 1967). Such reactions obey the law of mass action. When a labelled and unlabelled ligand are allowed to react with a known but limited amount of binding protein, they will compete for binding sites in proportion to their concentrations until an equilibrium is reached. Keeping the tracer amount constant, with the increase of unlabelled ligand the labelled ligand-protein complex will be decreased. A standard curve

can be obtained if one plots ligand-bound protein against unlabelled ligand added. In practice various types of plots are used (Midgely et al., 1969). From the standard curve, unknown samples can be quantified by comparison with the effect of a known amount of unlabelled compound.

2.5.4.1 RTA for Progesterone (PBG assay)

In this method progesterone was determined by using the natural protein, progesterone-binding globulin (PBG) (Heap et al., 1981) in pregnant guinea pig serum. PBG was first discovered when distinct differences from the cortico steroid-binding globulin (CBG) (Westphal, 1970) were observed (Diamond et al., 1969). Due to high affinity and specificity, PBG has been used for progesterone analysis (Tan and Murphy, 1974). From the affinity constants of the PBG complexes with more than a hundred steroids of the pregnane and androstane series, Blanford et al. (1978) reported that the progesterone molecule appeared to be in almost perfect contact with the binding site of PBG; introduction of most substituents decreased the binding affinity. Human CBG binds both cortisol and progesterone with similar affinity (Blanford et al., 1978); however, guinea pig CBG has about twenty times more affinity for cortisol than for progesterone (Mickelson and Westphal, 1980). Although progesterone binds to CBG and to albumin also, the effects of CBG and albumin were found to be negligible (Tan and Murphy, 1974), at the very high dilution of guinea pig

serum used in the PBG assay. Specificity data for guinea pig serum (Tan and Murphy, 1974) have been provided in Appendix 2.

For the standard curve, 0.0, 0.2, 0.4, 0.8, 1.2, 2.4, 4.8 ng, progesterone was pipetted in duplicate into glass tubes from the 10 ng/mL - 100 ng/mL stock solutions. Similarly, sample extracts were pipetted into sample tubes in duplicate or in triplicate. All the tubes (standard and samples) were arranged in a rack as follows :

- Two total counts (TC)
- one set of standards,
- one set of samples,
- 2nd set of samples
- 2nd set of standards.

This arrangement minimizes the influence of the time of charcoal contact. All tubes were air dried before adding protein-tracer solution (PTS). For PTS of 100 tubes, 130 μL of tritiated progesterone (from 50 μci/mL stock) was pipetted into an assay tube and air dried. To it, 1 mL of PO4 buffer (0.075 M, pH 7.5), 9 mL of gelatin water and 10 μL of guinea pig serum were added. This solution was freshly made up immediately before each assay. The PTS was then mixed gently by inversion after covering the mouth of the tube with a piece of parafilm. 0.10 mL PTS was added to each tube using a 5 mL Hamilton syringe.

The rack with assay tubes was then shaken to allow the proper mixing of PTS with the dried samples and standards.

Incubation was carried out at 45^{0}C for 5 minutes to redissolve progesterone in the PTS and at 4^{0}C for approximately 90 - 120 minutes. Following incubation, 1 mL dextran coated charcoal (DCC) solution was added to all tubes except the total counts; this was done at 4^{0}C. After 4 minutes, the tubes in the rack were shaken vigorously for 1 min in an automatic horizontal shaker at high speed at 22^{0}C and then centrifuged at 4^0 C at 2000 r.p.m. for 5 minutes to precipitate the charcoal and unbound progesterone. The tubes were placed back in the water bath at 4^{0}C. Supernatants 0.5 mL were transferred into counting vials. After adding 2 mL scintillation fluid (Optiphase) radioactivity was counted. An automatic program was used for plotting standard curve (Fig. 2.1). Sample values were read from the curve. Samples were corrected for dilution.

2.5.4.2 RIA'S for progesterone

The P4 concentrations in cultured samples were also evaluated by RIA employing two different antisera (As): (a) R4 and (b) P11-192. The specificity of R4 antiserum has been shown in our laboratory to be: progesterone: 100, 5α-dihydroprogesterone: 7, androstenedione: 0.1, corticosterone: 4.6 and deoxycorticosterone: 6. The specificity data for P11-192 antiserum have been provided in Appendix 3. Including the set up of standards and the addition of PTS, subsequent steps of incubation, charcoal addition, centrifugation and counting of

radioactivity for progesterone RIAs were similar to those of the PBG assay (Table 2.2).

2.5.4.3 RTA for testosterone (SHBG assay)

SHBG - a β-globulin in pregnancy plasma- has been used for the measurement of plasma androgens. Although SHBG has been used for the measurement of total plasma androgens without chromatographic purification (Horton et al., 1967; Murphy, 1968, 1969; Anderson, 1970), this is not applicable to asses individual placental androgens. To overcome this problem, placental androgens were measured after preliminary chromatographic separation, as described by Murphy (1988). The specificity data have been provided in Appendix 4.

The protocol for the SHBG assay was described previously from our laboratory (Murphy, 1970, 1968). The procedure is similar to that for the PBG assay. The details are indicated in Table 2.2. Unknown samples were read from the standard curve (Fig. 2.2) in terms of testosterone equivalents (TE) . Since the time of contact with adsorbent was critical, care was taken to keep this as uniform as possible throughout the assay. Not more than 24 samples with one set of standards were analyzed at a time by the SHBG assay.

2.5.4.4 RIA for 19-Nortestosterone

A single chromatographic purification in association with RIA was used for the specific determination of the relatively

low level of 19-NT in human placenta. The specificity data have been provided in Appendix 5. Standards and samples were incubated and processed in a similar manner similar to that for the SHBG and PBG assays (Table 2.2). A typical standard curve from which sample values were read, is shown in Fig. 2.3.

2.5.4.5 RIA for Androstenedione (A-dione)

Placental A-dione levels were measured by RIA assay kit after organic solvent extraction followed by column chromatography.

The procedure followed the instructions of the manufacturer of the androstenedione kit (Table 2.2). Two controls, level 1 and level 11, containing low (0.6-1.2 ng/mL) and high (4.0-8.0 ng/mL) A-dione concentrations in human serum were used. All the reagents were stored at 2-8^{0}C and used before their expiry dates. The mixing of kit components from different batch within an individual assay was avoided. The pellets remaining at the bottom of the assay tubes after decantation of the unbound fractions, were counted in a gamma counter (MGH) for one minute. A typical standard curve for the measurement of A-dione was shown in fig. 2.4.

2.5.4.6 RIA for 17β-Estradiol

The procedure routinely used in our laboratory for estradiol measurement was similar to that for the other hormones (Table 2.2). Polypropylene tubes (16 x 100 mm) were

used instead of glass tubes, due to the better recovery of ^{3}H-estradiol in this system (unpublished observation from our laboratory). A typical curve of estradiol standards vs bound radioactivity was drawn from my data (Fig. 2.5).

2.5.4.7 RIA for Estrone:

Estrone in sample extracts was measured by this method following exactly the same procedure as that for estradiol (Table 2.2).

2.5.4.8 Immunoradiometric assay (IRMA) for β-hCG

For the measurement of the hCG level in some of the culture samples, the IMMUNOCORP hCG IRMA kit employing coated tube assay was used. Although the beta subunits for the human glycoprotein hormones are distinct, the common amino acid sequences can cross react with some polyclonal antisera (As). The use of monoclonal antibodies (Ab) in the IMMUNOCORP coated tube IRMA hCG assay, eliminated this interference by providing a homogenous Ab with high affinity to hCG and low cross-reactivity with other glycoproteins (appendix 8).

The IRMA assay for hCG is a two-site immunoradiometric assay, generally called as a "sandwich" assay. The system utilizes a solid phase coupled monoclonal Ab (Ab coated tube) and a radiolabelled monoclonal Ab. The sample to be assayed is allowed to bind with the solid phase Ab during the incubation at room temperature. When labelled monoclonal Ab is added, it binds to the analyte to make the "sandwich". At the end of

incubation, the unbound radiolabelled Ab is removed by aspiration or decantation and washing. The radioactivity of the labelled Ab bound to the antigen on the solid-phase is quantitated in a gamma counter. A calibration curve is plotted from the known amount of standards and unknown samples are read directly from the curve.

Since the procedure is somewhat different than that described for the other assays used, the steps of IRMA for hCG are described briefly: Small aliquots (2-10 μL) of the samples were pipetted directly into the coated assay tubes. Repeated thawing and freezing of the samples were avoided. From each standard, 100 μL was pipetted at the bottom of the coated tube. Sample volumes (2-10 μL) were adjusted with porcine serum for standard volume (100 μL). All tubes were incubated for 45 minutes at room temperature. At the end of the incubation, the contents of the tubes were decanted and discarded. This was repeated after adding wash solution (4 mL/tube). After second wash and decantation, 100 μL of ^{125}I-mouse-anti-hCG was added to each assay tube and followed by gentle mixing and a second incubation at room temperature for 45 minutes. Except for the TC tubes, the contents from all tubes were decanted; to each tube 4 mL wash solution was added and decanted. The tubes were counted in a gamma counter. As for the other assays, the concentrations of the controls and unknowns were interpolated directly from the standard curve used for the measurement of hCG (Fig. 2.6).

2.5.4.9 Reliability of Hormone Analyses

The reliability of an assay depends on its sensitivity, specificity, precision and accuracy.

Sensitivity : The sensitivity of the assay is the detection limit i.e. the smallest amount of hormone that can be distinguished from zero with 95% probability. It is derived from the variability of the response at the zero dose level. For each hormone assay, it is calculated as 2 x SD of the reagent blank value as shown for the PBG assay (Table 2.3).

Table 2.3. Sensitivity of the PBG assay

[3H]-P4-bound in the absence of unlabelled P4 (mean ± SD) n=6	P4 measured at 6852 cpm (2 x SD at 'zero'level)
7262 ± 205 cpm	0.2 ng

Sensitivity of different steroid hormone-assays used in the present study was within the range of 16-40 pg (Table 2.5).

Specificity : The specific measurement of a steroid hormone depends on the affinity of the antiserum, its relative concentration in the biological sample, and the preliminary preparation of the sample (extraction, chromatography). Therefore, for the specific measurement of placental hormones

at relatively low concentrations (19-NT, A-dione, 5α-reduced steroids) chromatographic purification was done in addition to extraction before steroid hormone analysis. For any hormone analysis of cultured medium, the respective tissue blank was measured and subtracted. The specificity data for each assay are given in appendices 2-8.

Accuracy : The accuracy of the assay methods is evaluated by determining the recovery of known amount of the hormone added to samples (Table 2.5).

Precision : The precision of the assay is evaluated by the variability of the replicate measurements of the same sample within an assay (intra-assay variation). For PBG assay, intra-assay variation was calculated from 10 duplicate pairs at three concentration levels (Table 2.4).

Table 2.4. Precision of the PBG assay

Sample	no.of obs. (n)	P4 (ng/0.05 ml) mean±SD	C.V %
I	20	0.46±0.03	5.6
II	20	1.52±0.06	3.8
III	20	3.75±0.28	7.5

Intra-assay variations for other hormone-assays were less than 10% (Table 2.5).

Reproducibility : Reproducibility of the assay was tested by the inter-assay variation of a sample measured in duplicate in different assays of the same type. Inter-assay variation is always greater than intra-assay variation (Abraham, 1974). Although inter-assay variations of different assays were less than 20% (Table 2.5), for each set of experiments, the respective controls were included with samples within the same assay. Results were always compared with the controls in the same assay.

Table 2.2 Summary of hormone assays

Assay	Standards	Protein-tracer solution (for 100 tubes)	Incubation time	Separation of B and F
PBG	0, 0.2, 0.4, 0.8, 1.2, 2.4, 4.8 ng P4	130 µL *P4 (50 µCi/mL) 1 mL P4 buffer+ 9 mL GW + 10 µL GS	5 min - 45°C 90 min - 4°C	DCC
Anti P4 (R4)	0, 10, 20, 40, 80, 160, 320, 640 pg P4	52 µL *P4 (50 µCi/mL) 1 mL P4 buffer + 9 mL GW + 20 µL As (R4)	5 min - 45°C 90 min - 4°C	DCC
Anti P4 (P11-192)	0, 10, 20, 40, 80, 160, 320, 640 pg P4	20 µL *P4 (50 µCi/mL) 1 mL P4 buffer + 9 mL GW + 100 µL As (P11-192)	5 min - 45°C 90 min - 4°C	DCC
SHBG	0, 50, 100, 200, 400, 800, 1200 pg testosterone (T)	300 µL *T (10 µCi/mL) 9.8 mL SHBG buffer 200 µL LPS	5 min - 45°C 30 min - 4°C	DCC
RIA for 19-NT	0, 50, 100, 200, 400, 800, 1200 pg 19-NT	6 µL *19-NT (100 µCi/mL) 1 mL P4 buffer + 9 mL GW + 10 µL 19-NT As	5 min - 45°C 90 min - 4°C	DCC
RIA for estradiol (E_2)	0, 20, 40, 80, 120, 200 pg E_2	tracer solution: 50 µL *E_2 (50 µCi/mL) + 12 mL Buffer A. protein solution: 10 µL E_2 As + 9 mL buffer A	60 min - 37°C 30 min - 4°C	DCC

Table 2.2 cont...

RIA for estrone (E_1)	0, 20, 40, 80, 120, 200 pg E_1	tracer solution: 50 µL *E_1 (50 µCi/mL) + 12 mL buffer A. protein solution: 10 µL E_1 As + 9 mL buffer A	60 min - 37°C 30 min - 4°C	DCC
RIA for A-dione	0, 0.1, 0.3, 1,3, 10 ng/mL A-dione	per assay tube: 500 µL ^{125}I-A-dione (5 µCi/50 mL) + 100 µL As for A-dione	30 min - 37°C	double Ab PEG system
IRMA for β-hCG	0 ,5, 25, 50, 200, 500 IU/L β-hCG	per MoAb coated tube: 100 µL ^{125}I-hCG (10 µCi/5.5 mL)	45 min + 45 min - room temp.	double Ab

* = [^{3}H], GW = gelatin water, GS = guinea pig serum, B = bound and F = free radioactivity LPS = late pregnancy serum, T = testosterone, MoAb = monoclonal Ab
PEG = polyethylene glycol.

Table 2.5 Performance characteristics of hormone assays

Assay	Sensitivity	Precision (intra-assay variation %) (n)	Accuracy (% recovered) (n = 4)	Inter-assay variation % (n=6)
PBG	200 pg	6 ± 2 (30)	95 ± 7	12 ± 4
SHBG	20 pg	7 ± 1.5 (12)	82 ± 11	15 ± 7
RIA for 19-NT	16 pg	8 ± 1 (12)	89 ± 6	16 ± 5
RIA for A-dione	40 pg	4 ± 3 (30)	97 ± 5	6 ± 3
RIA for E_2	20 pg	6 ± 3 (12)	88 ± 9	13 ± 6
IRMA for hCG	1 mIU/mL	6.5±0.5 (30)	90 ± 8	10 ± 7

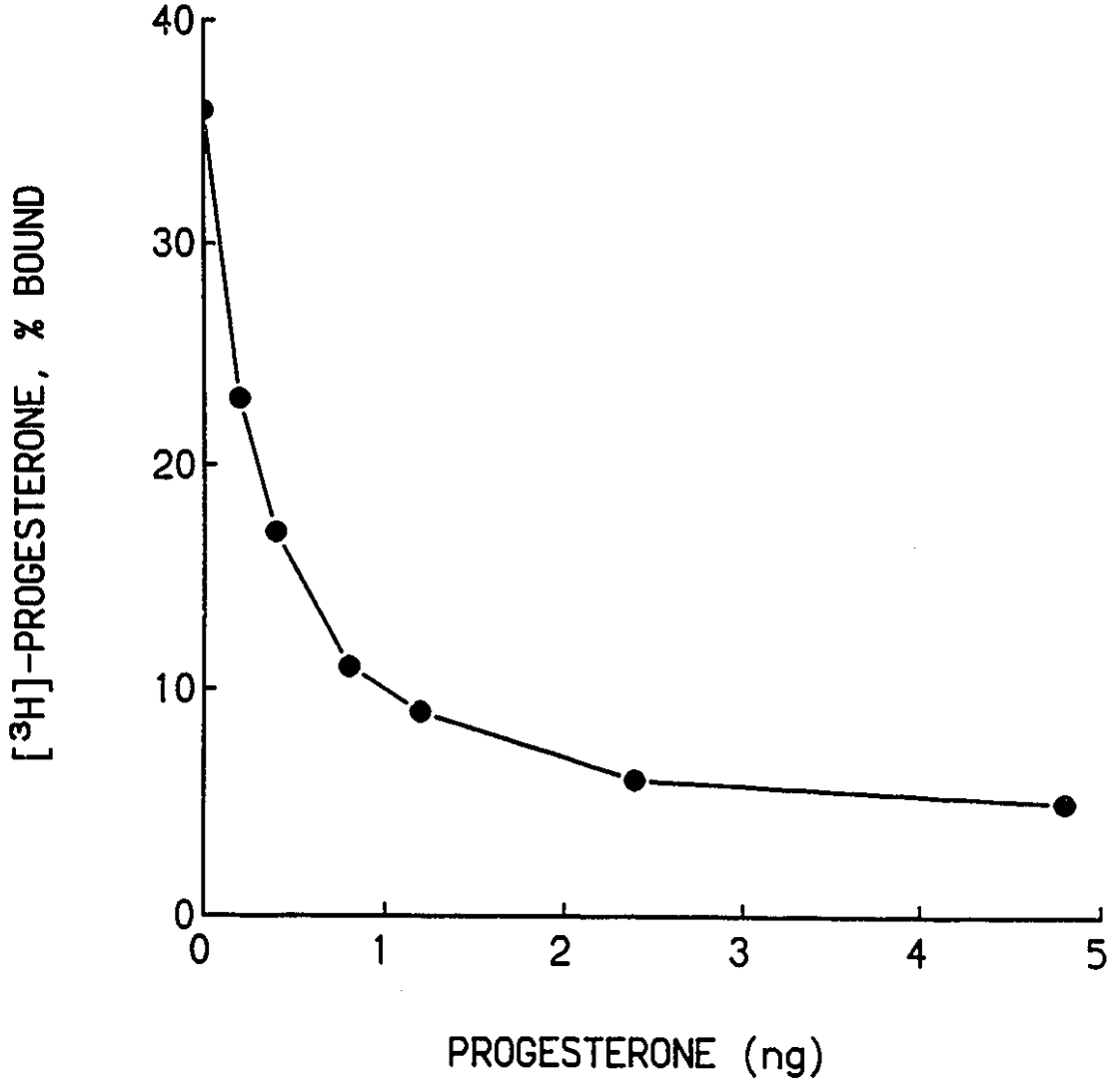

Fig. 2.1 Example of a standard curve for the radiotransinassay of progesterone in the guinea pig assay. Each point is the mean of duplicate determinations.

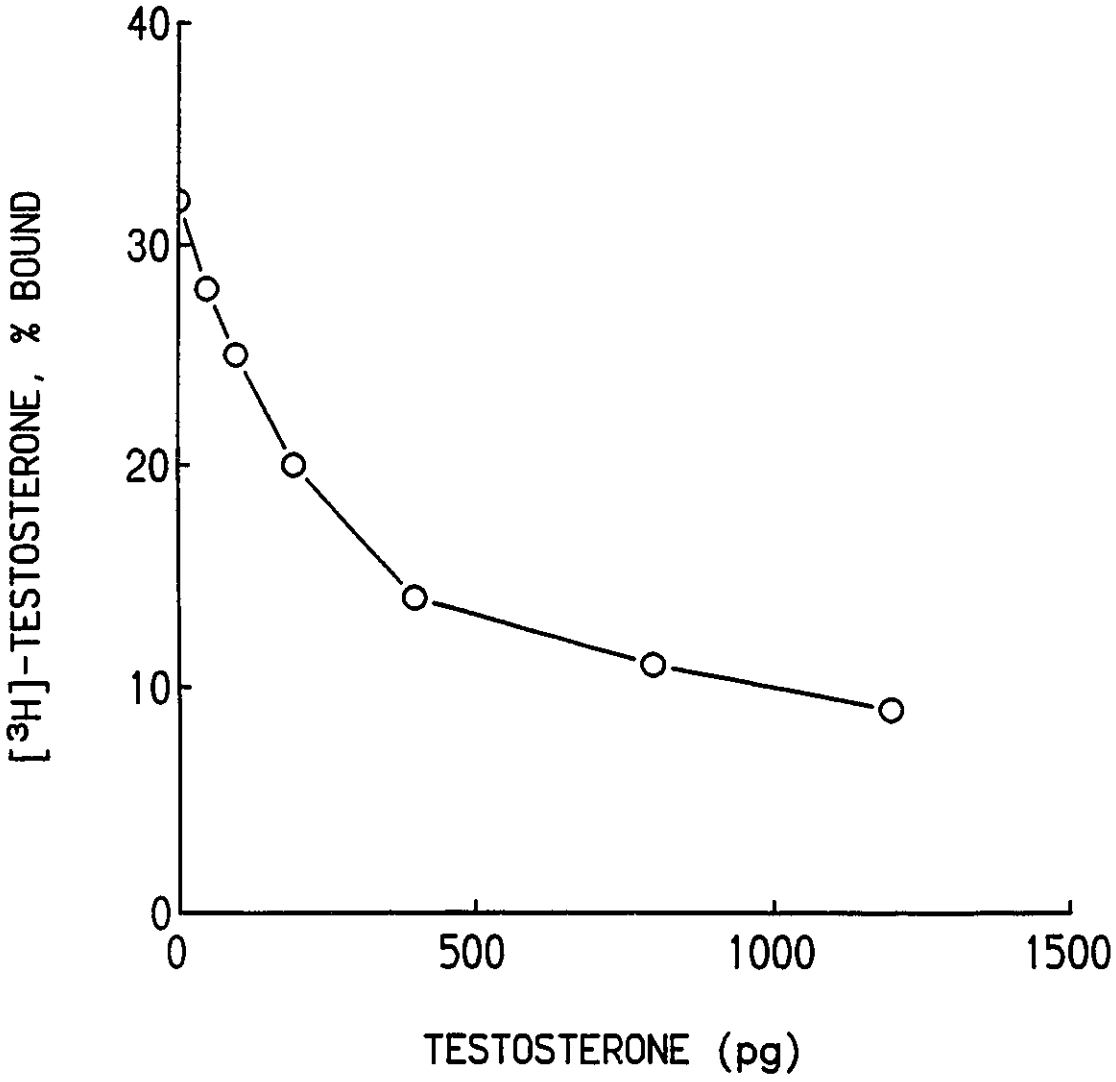

Fig. 2.2 Example of a standard curve used for the measurement of SHBG-bound activity (SHBG assay). Values are read off the curve and expressed as testosterone equivalents.

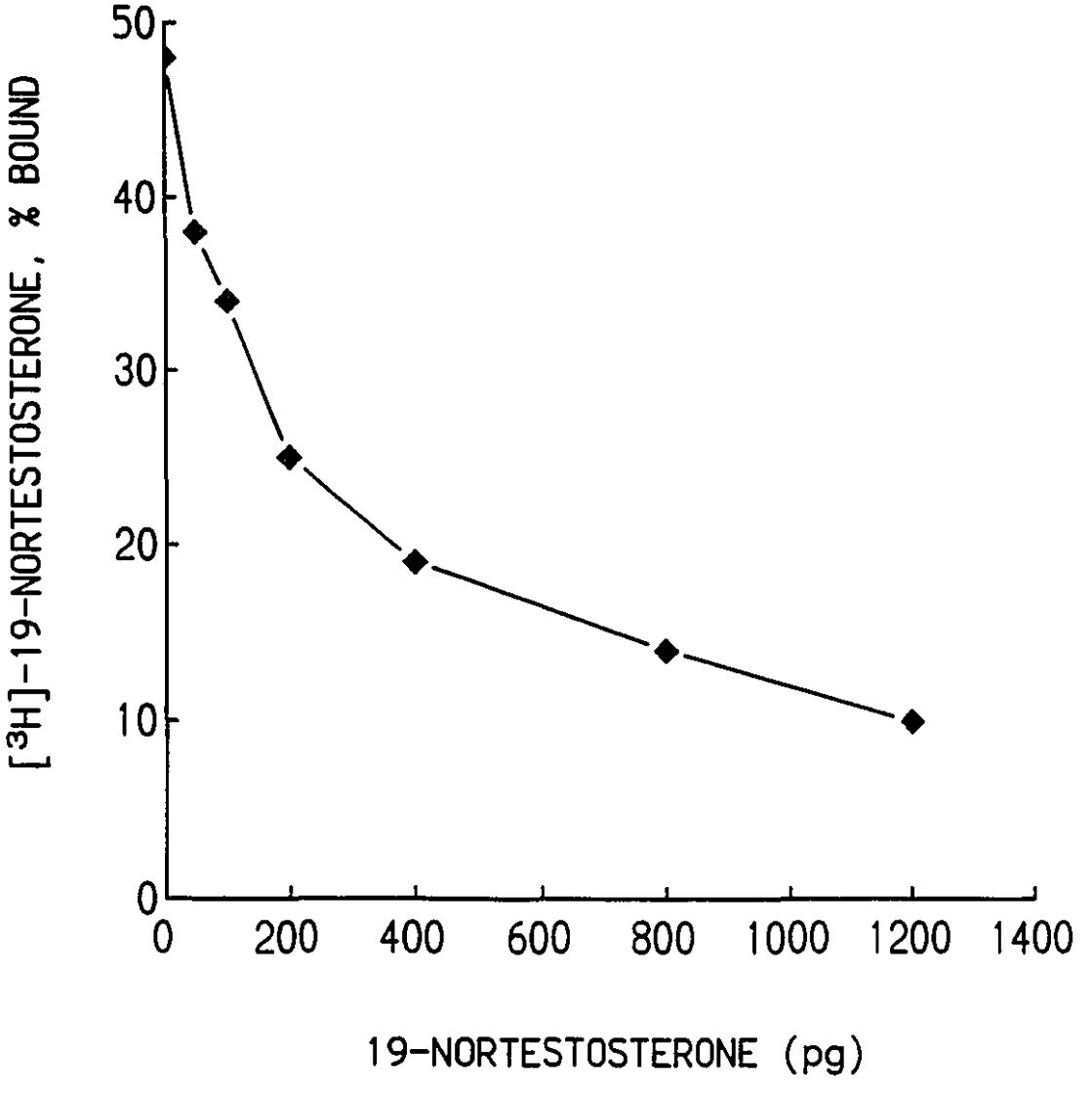

Fig. 2.3 Example of a standard curve used for the radioimmunoassay of 19-nortestosterone.

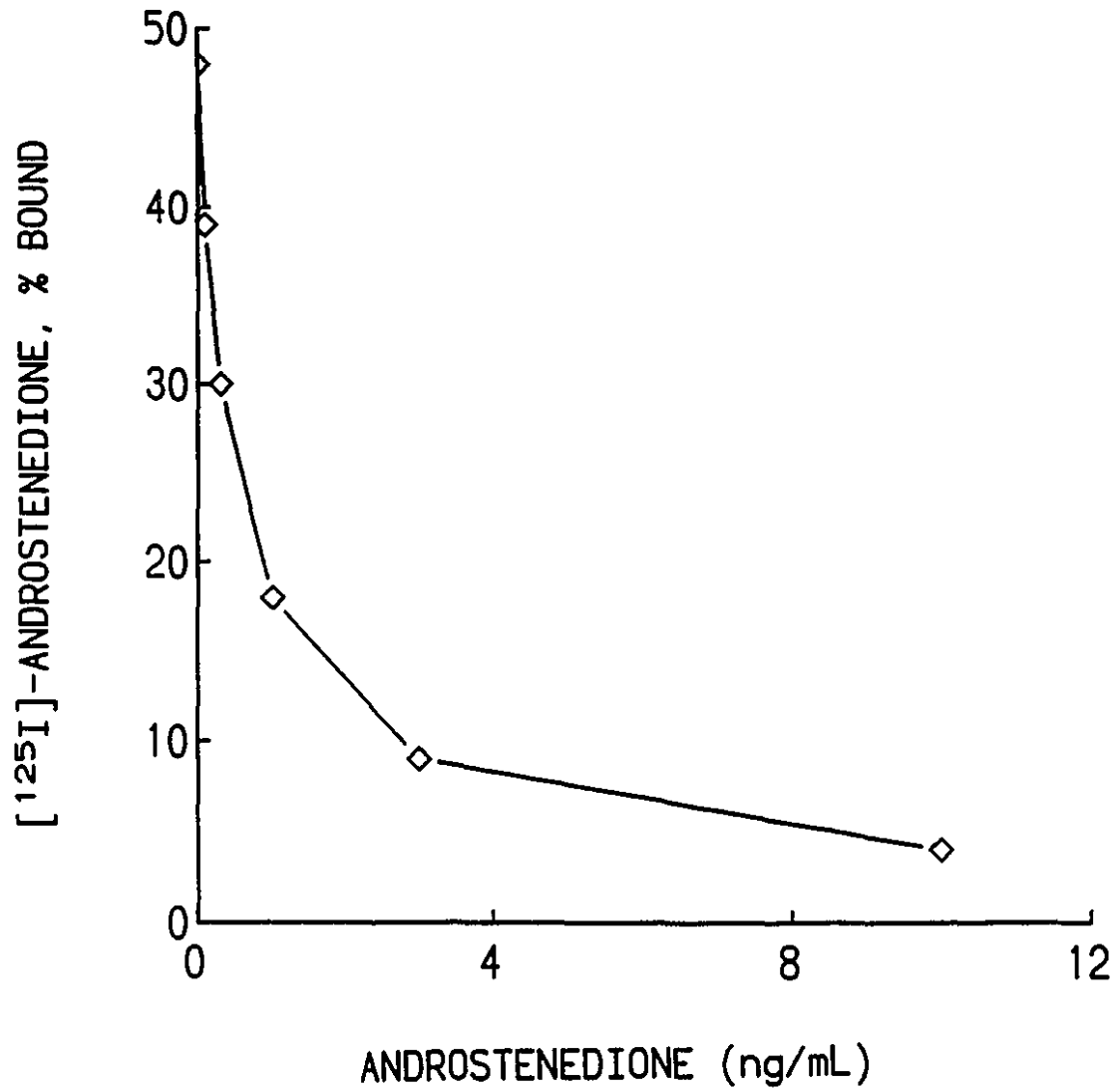

Fig. 2.4 Example of a standard curve used for the radioimmunoassay of androstenedione.

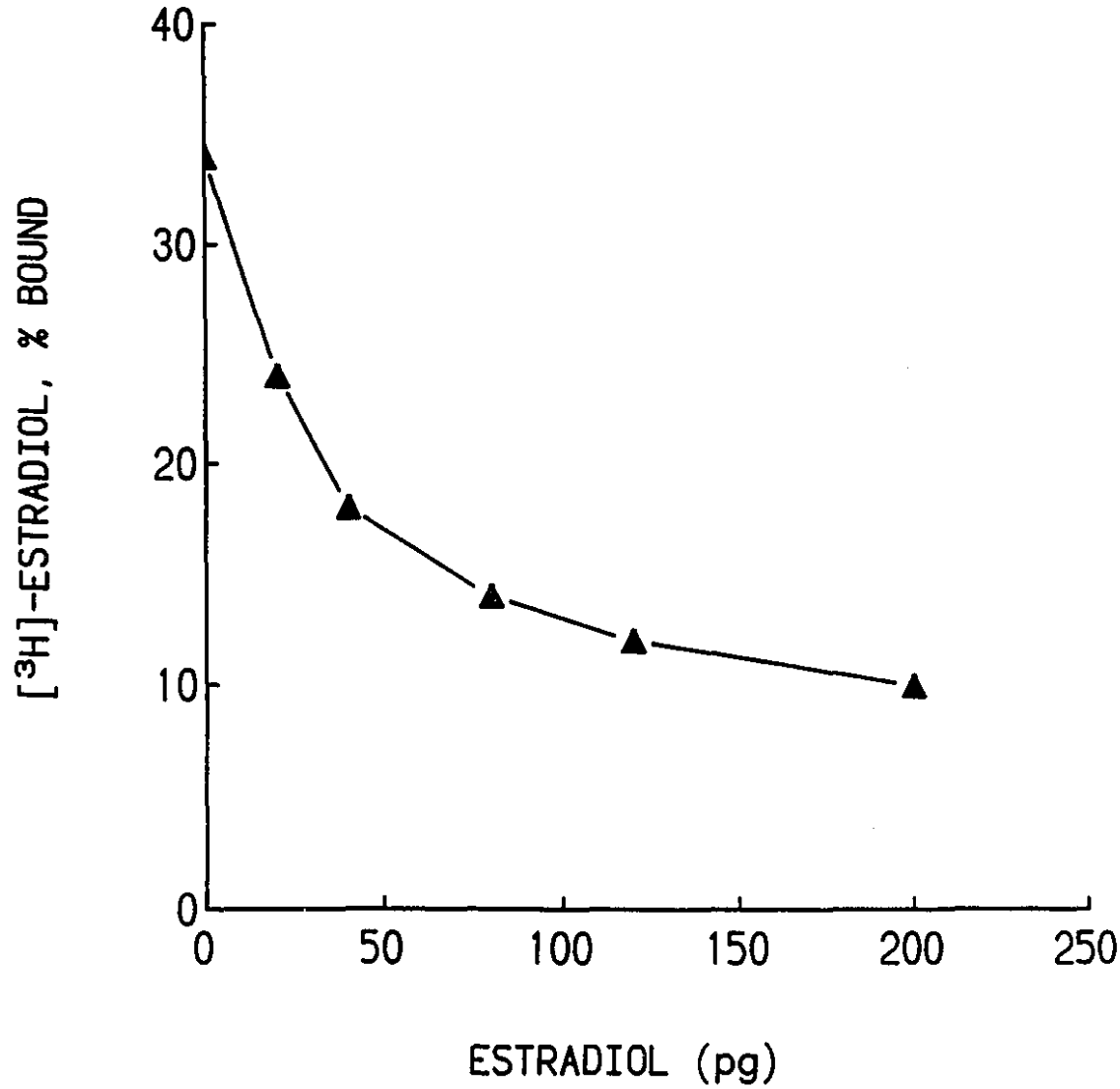

Fig. 2.5 Example of a standard curve used for the radioimmunoassay of estradiol.

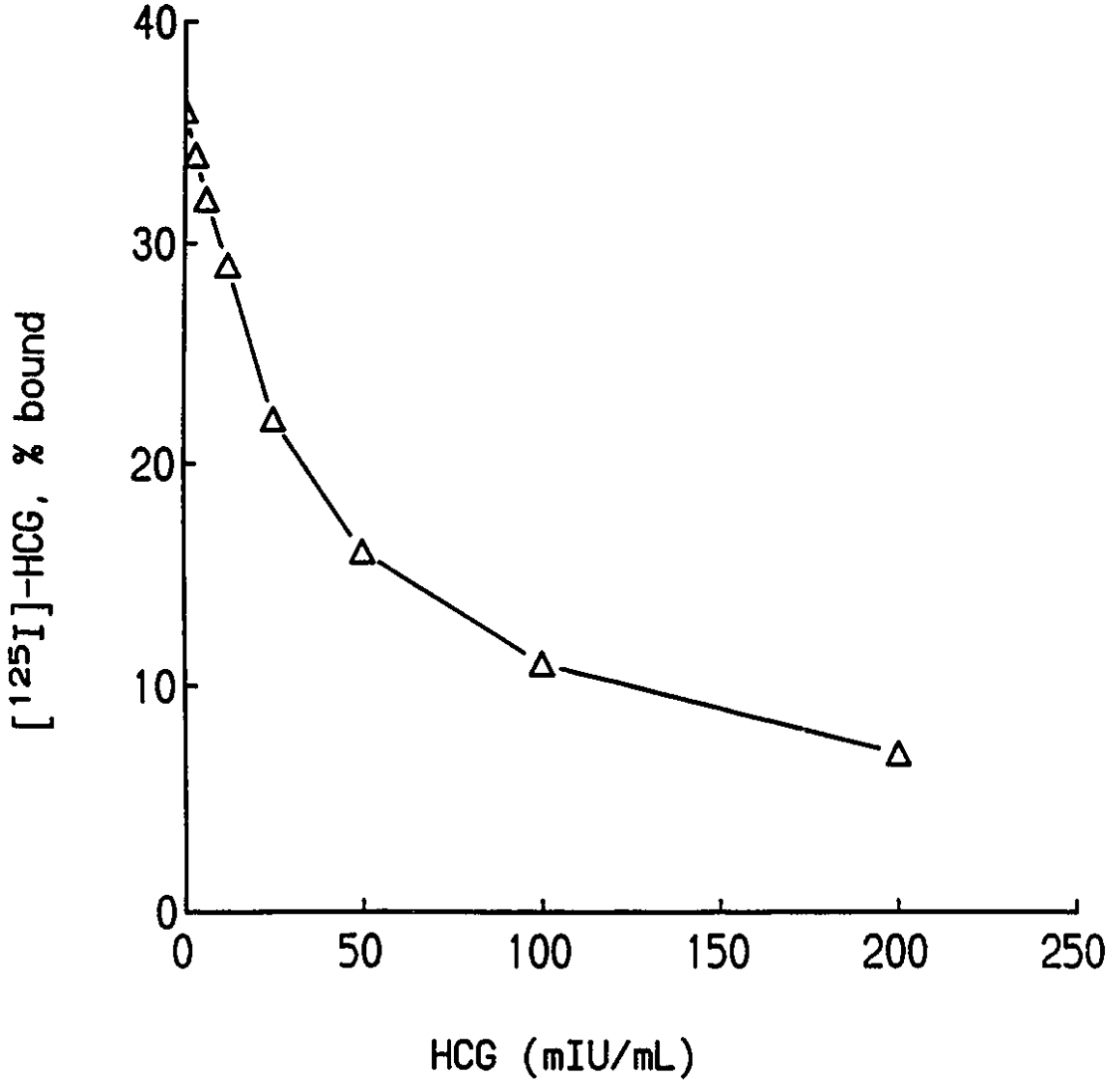

Fig. 2.6 Example of a standard curve used for the measurement of hCG.

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CHAPTER III

IN VITRO STIMULATION OF PLACENTAL PROGESTERONE PRODUCTION BY 19-NORTESTOSTERONE AND C_{19} STEROIDS IN EARLY HUMAN PREGNANCY[1]

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Key words: early human placenta, progesterone, 19-nortestosterone, C_{19} steroids.

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3.1 ABSTRACT

To explore the regulatory mechanism at the critical period of the luteal-placental shift, the effects of various steroids and peptides on the production of progesterone (P4) by placental explants at 7-10 weeks were studied. Androstenedione increased P4 production 3-fold at a concentration of 1 μM and more than 20-fold at 18 μM. 19-Nortestosterone (1-18 μM) stimulated P4 production 10-100 fold. 5α-Androstane-3β,17β diol (1-18 μM) stimulated P4 production about 2 to 5-fold while its 3α isomer (1-6 μM) increased it 2-fold. Estrone, estradiol and estriol up to a concentration of 36 μM had no effect. DHAS (to 36 μM), androst-5-ene-3β,17β diol (1 to 6 μM), 5β-androstane-3α,17β diol (1-6 μM), and DHT (1-12 μM) had no effect. Cortisol and dexamethasone (1-12 μM), hCG (20,000 IU/L), GnRH (4 μM) and ACTH 1-24 (20 μM) also had no effect. Thus, of all the compounds tested, only 19-nortestosterone and, to a lesser extent, androstenedione, 5α-androstane-3β,17β diol, and 5α-androstane-3α,17β diol stimulated P4 production in early pregnancy; at term, only 5α-androstane-3β,17β diol was stimulatory. 19-Nortestosterone was found to be less efficiently aromatized compared to other androgens; since it is also known to be present in blood from pregnant women and thought to be made in the placenta, the stimulation observed may be a paracrine effect. These observations suggest that C_{18} and C_{19} steroids may be important in the regulation of P4 synthesis by the human placenta in early pregnancy.

3.2 INTRODUCTION

Progesterone has long been known to be an important determinant for the maintenance of human pregnancy. In the human species, corpus luteum production is supplemented by placental production from about 7-8 weeks so that the corpus luteum becomes dispensable at about 12 weeks of gestation when the placenta becomes the major source of progesterone (1). However, little is known about the placental regulation of progesterone at this critical time.

Although Ogino et al (2) concluded that the increase in placental weight throughout pregnancy accounts for the increase in maternal plasma levels of progesterone, the correlation between the progesterone production rate, the maternal plasma progesterone levels and the placental weight is poor. The curve for placental weight increase is much steeper from 6 - 24 weeks than either of the other two parameters, i.e. the placental weight quintupled over this period while the progesterone production rate increased only about 10% and the plasma level approximately doubled. These observations suggest that the small mass of placenta in the early weeks may be producing more progesterone per unit weight.

The effects of estrogen and androgen on placental progesterone synthesis have been reported by many investigators but the reports are not consistent. Estrogen at "physio-

logical concentrations" (0.09 - 0.9μM) was shown by Genti-Raimondi et al. (3) to stimulate progesterone formation from pregnenolone by human term placental fragments incubated in vitro; however, inhibition was observed at higher concentrations. Inhibitory effects of estrogen on the 3β-hydroxysteroid-dehydrogenase (3β-HSD) enzyme activity were reported by others (4,5). More recently Pepe and Albrecht (6,7) showed that progesterone formation is decreased by the administration of the anti-estrogen MER-25 and 4-hydroxy-androstenedione in baboon pregnancy. Similar findings were also demonstrated in monolayer culture of human placenta collected during late gestation (8). However, in all of these studies, estrogen did not have any direct effect on progesterone production.

Branchaud et al. (9), using human placental monolayer culture, and Grimshaw et al. (10), in short term incubations of human term placental cell suspensions, also showed that progesterone formation was not altered by estrogen. Branchaud et al. (9) also showed that progesterone production was inhibited by high concentrations (10 μM) of dehydroepiandrosterone (DHA), while Grimshaw (10) showed inhibition by DHA, estrone, androstenedione and testosterone. Due to its easy availability, most of the above studies have been conducted in placenta obtained at term.

At present, our knowledge of progesterone regulation at the critical stage of the luteoplacental shift is limited and incomplete. The objective of the present study was to

investigate the relationship of various steriods and peptides to progesterone production in placenta obtained at 7 to 10 weeks gestation.

3.3 MATERIALS AND METHODS

After obtaining informed consent, placental samples [7 to 10 wks, dated from the day of the last normal menstrual period and confirmed by measurement of foot length (11)] were obtained from patients undergoing elective abortion. The tissues were collected in saline at the time of dilation and evacuation, performed in the Pregnancy Termination Unit of the Montreal General Hospital. Term placenta was obtained at normal delivery.

Powdered Ham's F-10 culture medium and fetal bovine serum were purchased from Grand Island Biological Company (Burlington, Ontario). Amphotericin B was obtained from Squibb (Montreal), penicillin G from Glaxo Laboratories (Montreal) and gentamicin sulfate from Schering (Pointe Claire, Quebec). Falcon plastics (Los Angeles, CA) provided the plastic culture dishes (60 x 15 x 2 mm). Tissue supporting grids (2 x 1.5 cm) were from Johnson wire works (Montreal, Quebec). The glass-redistilled (analytical grade) diethyl ether was purchased from American Chemical Company (Montreal, Quebec).

Unlabelled steroids, hCG and monobutyryl cAMP were obtained from Sigma Chemical Company (St. Louis, MO).

Steroids were dissolved in redistilled ethanol and stored at -10^{0}C. ACTH was from Organon Canada Ltd., West Hill, Ontario and GnRH was from Ayerst Laboratories, Montreal. Radioactive steroids namely, [2,4,6,7-^{3}H(N)]estradiol, SA 115 Ci/mmol, [2,4,6,7-^{3}H(N)]estrone, SA 87.8 Ci/mmol, [7-^{3}H(N)]DHAS, SA 35 Ci/mmol and [1,2,6,7-^{3}H(N)]progesterone, SA 112 Ci/mmol were obtained from New England Nuclear Corporation (Boston, MA); these were diluted with ethanol to a concentration of 50 μCi/mL and stored at -10^{0}C. Counting was done in Optiphase (Fisher Scientific Co., Montreal, Quebec).

Tissue processing

Tissues were kept on ice until processed within one to two hours of removal. After rinsing several times with cold saline, placental villi were dissected with sharp scissors and scalpel. Ten explants, about 1 mm^3 each - five on each of the two supporting grids - were placed in a culture dish containing 3 mL of control medium composed of Ham's F-10 + fetal bovine serum 10% + antibiotics (penicillin G: 200 IU/mL; amphotericin B: 5 μg/mL; gentamicin sulfate: 40 μg/mL). Thus each culture dish contained approximately 10 mg tissue.

Incubation

Culture dishes were incubated in a humidified atmosphere at 37^{0}C under 5% CO_2 in air. After initial 24 hr incubation period in control medium, enriched media were added usually on

day 2. The media were collected at 24 hr. Spent media were stored at -20^{0}C for subsequent hormone analysis.

Extraction

Prior to assay, steroid hormones were extracted into five volumes of diethyl ether. Extraction recovery of tracer hormones was 90% to 95%. Each extract was transferred to an assay tube and evaporated to dryness.

Hormone assays

The progesterone concentrations of the extracts were estimated by competitive protein binding (12) to pregnant guinea pig serum (McGill Animal Research Centre, Montreal) which contains a protein with high affinity and specificity for progesterone (13) (Table 3.1). Briefly, 0.1 mL protein-tracer solution [late pregnancy guinea pig serum or plasma 10 μL; tracer progesterone 130 μL (50 μCi/mL); 1.0 mL 0.075M phosphate buffer, pH 7.5; 9.0 mL gelatin water (0.5 gm/L)] was added in duplicate to tubes of each extract and standard tubes containing 0, 0.2, 0.4, 0.8, 1.2, 2.4, and 4.8 ng progesterone. The rack containing all the tubes was incubated at 45^{0}C for 5 min, then at 4^{0}C for about 90 to 120 min. While still in the cold bath, 1.0 mL charcoal solution [0.25 gm/L Norit A charcoal, 0.25 gm/L dextran] was added to each tube; the tubes were left standing in the bath for 4 min, then centrifuged at 4^{0}C for 5 min. Supernatant 0.5 mL was transferred to a

counting vial, and 2 mL Optiphase added. The tubes were shaken and counted to 10,000 cpm. The time in min/10,000 cpm was plotted against the progesterone in ng, and the values of the unknowns were read off the curve.

Because of the possibility that cross-reacting materials other than progesterone and the steroid added might be produced during the incubation, a series of 72 representative samples was also assayed using an antibody raised in our laboratory to 3-CMO-progesterone, which has a different profile of specificity (Table 3.1). Values obtained in the absence of added steroid (n = 16) were identical (54.6 ± 23.2 S.D. μmol/L/day for guinea pig, 54.6 ± 23.8 S.D. μmol/L/day for the antibody). The mean values for each group (i.e. each steroid assayed 4-8 times at 1 and 6 μM correlated well ($r = 0.985$, $P \leq 0.0001$), and did not differ significantly for the two methods. In addition, 10 samples were compared before and after chromatography on Sephadex LH-20 (37 cm column; elution solvent heptane 50, methylene chloride 50, ethanol 1) (14). The values are Representative samples (2 basal, 2 at the 6 μmol concentration for each of the steroids which increased progesterone production).

17β-estradiol and estrone were determined by radioimmunoassays in similar fashion using highly specific antibodies kindly provided by Dr. Hamish Robertson (Ottawa, Ontario).

For each culture, control and experimental dishes were prepared using the same placenta. For each concentration of

hormone used, samples without tissue were included as controls to determine the cross-reactivity of the hormones in the assay. The values in the absence of tissue were subtracted from those obtained in the presence of placental tissue. Samples from a given experiment were assayed simultaneously to avoid inter-assay variation, which is about 15%, for both estrogen and progesterone assays. Intra-assay variations for both were 9% or less. Hormone concentrations of sample media were expressed as nmol/L unless otherwise indicated. To compensate for the variation from culture to culture, data in some figures are given as a percentage of the control value i.e. that obtained in the absence of added hormones.

Sulfatase activity in early placenta

Fresh placental tissue 0.5 gm was homogenized in 2 mL 0.9% saline and incubated in the presence of 10,000 cpm tritiated dehydroepiandrosterone sulfate (DHAS) for 2 hr at 37^0C. The incubate was then extracted with 5 volumes of ethyl acetate, and the two phases separated and counted.

Statistical analysis

From the observations of the replicate experiments, means and standard errors (SE) were calculated. The significance of the difference between control and experiment was determined by using Student's t-test. The level of probability accepted as statistically significant was $P < 0.05$.

3.4 RESULTS

Time course of progesterone production

A preliminary experiment (Fig. 3.1) was done to assess whether the progesterone content of cultured medium is due to synthesis or release of preformed progesterone in placenta. The amount of progesterone secreted during during incubation 2, 4 and 24 hrs after the tissue was put into culture was measured. The endogenous concentration of progesterone was 6 ± 2 μmol/kg, mean ± SE, n = 4. During incubation there was an increase of progesterone in the culture medium in excess of the endogeneous content in fresh placenta. A significant amount of progesterone was secreted by 4 hrs incubation (60 ± 4 nmol/L, n = 4, $P < 0.05$) and increased only slightly by 24 hr (74 ± 8 nmol/L, n = 4). For convenience, we usually measured the 24-hr secretion of progesterone.

Basal progesterone in presence of cycloheximide

In the presence of 1 mM cycloheximide, progesterone production fell from 129 ± 8 to 13 ± 6 nmol/L (n = 3) ($P \leq 0.001$).

Progesterone production from exogenous precursors

Under control conditions, i.e. in the absence of added precursors, progesterone production was 86 ± 10 nmol/L. Pregnenolone at a concentration of 1 μM was converted into 395

± 18 nmol/L progesterone (n = 6), a 40% conversion. When the concentration of pregnenolone was increased 5-fold, production of progesterone only doubled (745 ± 74 nmol/L (n = 5).

Effect of 5α-androstane-3α,17β diol (3α-Diol)

Progesterone production was assessed in the presence of the 3α isomer, 3α-Diol (Fig. 3.2). At the two concentrations, 1 and 6 μM, progesterone production was stimulated, respectively, to 200 ± 13% ($P \leq 0.05$) and 218 ± 18% ($P \leq 0.05$) of control.

Effect of 5α-androstane-3β,17β diol (3β-Diol)

Fig. 3.2 illustrates the stimulatory effect of 3β-Diol on progesterone production. With the addition of 1-36 μM, 3β-Diol augmented progesterone accumulation up to 14-fold (265% ± 25% - 1443% ± 161%). In comparison to control, significant differences were observed at all concentrations ($P < 0.05$ to $P < 0.01$).

Effect of androstenedione (A-dione)

Dose-dependent stimulation of progesterone production in culture by androstenedione is shown in Fig. 3.3. The results are expressed as a percentage of control. When androstenedione was added at concentrations ranging from 1-36 μM, formation of progesterone was increased from 345% ± 80% to 6380% ± 280% of control, a significant difference ($P < 0.05$ to $P < 0.01$), at all the concentrations.

Effect of 19-nortestosterone (19-NT)

Increasing concentrations of 19-NT also caused a dose-dependent stimulation of progesterone production (Fig. 3.3). The increase of progesterone production was about 10, 35 and more than 100 times control at the concentrations of 1, 6 and 18 μM, respectively ($P < 0.05 - 0.001$).

Validation of results by chromatography

To confirm the identity of the material measured as progesterone, 10 samples were compared before and after chromatography on sephadex LH-20 (Table 3.2). The values agreed within experimental error and the correlation of values before and after chromatography was excellent ($r = 0.99$, $P \leq 0.001$).

Comparison of the effects of C_{18} and C_{19} steroids on progesterone production

Comparing the above results, by far the most effective stimulator was 19-NT. Androstenedione had a lesser effect, while stimulation by the androstanediols was minimal. The order of effectiveness was thus 19-NT > A-dione > 3β-diol > 3α-diol.

Time course study for A-dione and 19-NT

Further experiments were conducted to study the time course of progesterone stimulation by androstenedione and 19-

nortestosterone (Fig. 3.4). When the explants were incubated for 2, 4 and 24 hrs in presence of androstenedione (1 μM), progesterone production was increased 1.3 times (32 ± 3 nmol/L), 2 times (60 ± 10, $P < 0.05$) and 2.7 times (200 ± 12, $P < 0.05$), respectively, over the control. At all the incubation periods, the addition of 19-NT (1 μM) resulted in a greater progesterone concentration than that in control dishes. It enhanced progesterone production about 4-fold by as early as 2 hours and with a greater effect of about 6-fold by 24 hr (420 ± 80 nmol/L, $n = 4$, $P < 0.01$).

Effect of steroids stimulatory in early pregnancy on term placenta

In term placental culture (Fig. 3.5), 3β-Diol (1-6 μM) was found to enhance progesterone production 4-7-fold, $P \leq 0.05$); whereas the other steroids which were shown to stimulate progesterone production in early pregnancy (19-NT, A-dione, 3α-Diol) had no effect at the same concentrations.

Aromatization of 19-NT and C_{19} compounds

Since both 19-NT and androstenedione are potential substrates for placental aromatization, the degree to which they are aromatized was explored along with another substrate (androst-5-ene-3β,17β diol) by measuring estrone and 17β-estradiol (E_2) production in culture (Fig. 3.6). Estradiol production by androstenediol, androstenedione and 19-nortest-

osterone at 1 μM was 116 ± 9 ($P \leq 0.01$), 30 ± 2 ($P \leq 0.05$) and 3 ± 0.2 nmol/L, respectively. Thus, androstenediol was aromatized most effectively (about 12%), androstenedione (3%) and 19-NT least (0.3%); i.e. 19-NT was aromatized only 1/10 as effectively as androstenedione, and only 1/36 as effectively as androstenediol. The 5α- and 5β- androstanediols, lacking the 5-ene or 4-ene structure, did not increase estradiol production above the control (data not shown). When estrone was measured, the relative degree of aromatization of androst-5-ene-diol (18%), androstenedione (5.8%) and 19-NT (1.4%) was similar to that found for estradiol.

Comparison of the effects of 19-NT and C_{19} steroids on progesterone and estradiol production in culture

As summarized in Table 3.3, 19-NT, which is the most effective stimulator of progesterone production, was the least effective with respect to estradiol production in culture when compared to the other compounds. Androstenedione had moderate effects on both estradiol and progesterone production. Androstenediol (androst-5-ene-3β,17β-diol), having no effect on progesterone production, was the most effectively aromatized. The androstanediols, which enhanced progesterone production, did not alter estradiol content.

Compounds without effect on progesterone production in culture (Table 3.4)

In our culture system, neither estrone nor estradiol (up to 36 μM) had any effect on progesterone production. Nor was there any alteration of progesterone production in culture when explants were treated with the aromatase inhibitor, 4-hydroxyandrostenedione. Similarly, dehydroepiandrosterone sulfate, dihydrotestosterone and testosterone were without effect at the concentrations used. In contrast to 5α-reduced compounds, a 5β-reduced isomer (5β-androstane-3α,17β diol) as well as androstenediols at 1-6 μM did not show any effect on progesterone production. Cortisol and dexamethasone, hCG, GnRH and ACTH had no apparent effect. We also did not observe any effect of a cAMP analog (monobutyryl cAMP).

Sulfatase activity in early placenta (Table 3.5)

The conversion, shown in Table 3.5 for four different placentas, averaged 84%, indicating that this enzyme is present from at least 6 weeks gestational age.

3.5 DISCUSSION

Our explant culture system provided a useful model for investigation of in vitro hormonal regulation, since there is a net synthesis of progesterone in culture over the endogenous content of placental progesterone. The initial delay of 2 hr to have significant progesterone synthesis might be due to the time taken for adaptation of the tissue to the in vitro culture environment or due to insufficient interaction of substrate with enzyme as suggested by Ogino et al. (2). The significant decrease in basal progesterone production in the presence of cycloheximide further supports the synthesis of progesterone rather than release of preformed hormone in culture. Similar to our results, 40% to 60% conversion of radio-labelled pregnenolone to progesterone has been reported previously (15).

The time course study with 19-nortestosterone showed a rapid production of progesterone in cultured media within 2 hr of incubation, which increased with incubation time, also confirming synthesis and not merely release.

In this study we observed dose- and time-dependent stimulation of progesterone by androstenedione. In contrast, androstenedione was shown to decrease progesterone formation in term placental monolayer culture (10). This difference may be related to gestational age or to the culture method used. On the other hand a stimulatory effect of progesterone by

androstenedione has been reported in bovine ovarian follicles (16). There are a number of reports of progesterone stimulation by androgen (17,18) in granulosa cells but none in placenta. The stimulatory effects of 5α-reduced androstanediols on progesterone production have not previously been reported. However, progesterone formation was found to be stimulated by a 5α-reduced C_{21} compound (5α-pregnane-3,20-dione) (10) but not by the 5β compound, pregnanediol. These authors speculated that the stimulatory effect was due to end-product inhibition of progesterone metabolism, possibly restricted to the 5α isomers. In our study both 5α-androstanediols, but not the isomer 5β-androstane-3α,17β diol, were stimulatory, also suggesting that the effect is stereospecific for the 5α form.

Among all the compounds tested here and of those reported in the literature (3,4,8-10), 19-NT is the most potent stimulator of progesterone production. Although the physiological relevance of this finding is not completely understood, there is in the literature to support this concept. Recently, Reznik et al. (19) documented the presence of 19-NT in maternal serum as a naturally occurring steroid. In their study the plasma level of 19-NT was found to rise throughout human pregnancy to levels as high as 60 pg/mL. They determined 19-NT by radioimmunoassay and validated the results by gas chromatography and mass spectrometry. They suggested that 19-NT may be of placental origin. Since the formation of 19-

NT and 19-norandrostenedione from the metabolism of testosterone by baboon placental microsomes has been reported (20), it is reasonable to expect a similar occurrence in human placenta. In 1960, Short (21) reported the presence of 19-norT and 19-norandrostenedione in mare follicular fluid, and recently Dehennin et al. (22) identified these steroids in human follicular fluid.

Our own studies of placental concentrations (Begum-Hasan and Murphy, in preparation) suggest that levels of 22-30 nmol/kg 19-NT and 80-100 nmol/kg androstenedione are present in early pregnancy. While these are 1/10 to 1/30 of the lowest concentration used here (1 μM) it is accepted by those who work with tissue cultures that approximately 10 times the physio- logical concentration of many substances is necessary to obtain corresponding effects in vitro.

Very recently, Osawa et al. (23) have reported increasing serum levels of 19-hydroxyandrostenedione through pregnancy to 1500 pg/mL, a high level in umbilical vein (2000 pg/mL) compared to umbilical artery (110 pg/mL), and a high term placental content (16.3 ng/gm). The presence of another possible precursor of 19-NT, namely, 19-hydroxyprogesterone, and possibly also 19-norprogesterone, has also been reported (24). All of the above observations lend strong support to the possibility of the presence of 19-NT as a natural compound in placenta.

The mechanism by which 19-NT enhances progesterone

accumulation is not clear at the present time. It may involve the stimulation of biosynthesis of progesterone or inhibition of its metabolism. The latter seems unlikely since our own data (Begum-Hasan and Murphy, in preparation) and those of Winkel et al. (25) showed that there was minimal metabolism of progesterone in culture.

It is well recognized that androstenedione and 19-NT are potential substrates for the aromatase system of human placental microsomes (26,27). In accord with our results it was demonstrated previously that the rate of aromatization of 19-methylandrogens is far more effective that that of 19-norandrogens (28,29). It was found by many investigators (30-32) that 19-norT is not a preferential substrate for human placental microsomal synthetase at term. Similar findings have been reported for human ovarian follicles (22). Our observation of 19-NT aromatization in early placenta (6-10% that of androstenedione) is comparable with that of Ryan (30) and of Canick and Ryan (31) who reported that the extent of 19-NT aromatization was only 1/5th that of androstenedione in late placenta. The extent of 19-NT aromatization reported in the present study (0.3%) is in good agreement with that of Fronckowlak et al. (32) who obtained 0.5 to 1%, using a purified human placental aromatase enzyme preparation. The above findings suggest that 19-NT accumulates enough to have its own physiological role in the placenta.

It is suspected that the biosynthesis of 19-norandrogen

may be related to a non-aromatizing C-19 desmolase similar to one documented for d[illegible] adrenal (33). The source of the precursor for 19-NT formation is not known. It seems likely that dehydroepiandrosterone sulfate (DHA), present in high concentrations in maternal blood (34), and rapidly converted to DHA by placental sulfatase in early (our data) as well as late (35) placenta, provides precursors for 19-NT formation in the placenta. However in our study we did not find any effect of DHAS on progesterone production. This failure may be related to the use of insufficient concentrations of DHAS in culture or there may be another precursor.

At term, progesterone production was stimulated only by 3β-Diol, not by 19-NT, A-dione or 3α-Diol. This suggests a temporal relationship of progesterone regulation with the local concentrations of progesterone stimulated steroids in the human placenta.

Since estrogen did not have any direct effect on progesterone production in our culture system and those of others (7-9), it seems likely that stimulatory effects of androstenedione and 19-NT are more directly on progesterone, instead of secondarily through estrogen. Our results are consistent with those of Wunsch et al. (8) who did not find any influence of DHA on progesterone production in human term placental monolayer culture. It was reported earlier (36) that DHT had no effect on progesterone in term culture, in accord with our observation. Although dexamethasone was found to decrease

progesterone synthesis in rat placenta (37), we were unable to show a similar effect in early human placenta, possibly due to a species difference. It was proposed by Caritis et al (38) that β-adrenergic receptor stimulation can enhance placental progesterone production by increased intracellular cAMP production. The stimulatory effect of cAMP on progesterone production was also reported in term placental cell culture (39). However, in our study with early placenta, progesterone formation was not increased in the presence of a cAMP analog. This discrepancy may be related to the difference of placental age or culture method, or possibly to the particular analog used.

The importance of ACTH on adrenal steroidogenesis is well known (40) but a similar effect is not present in placenta. Siler-Khodr et al. (41) reported that progesterone release in culture from mid-gestational placenta was suppressed by a GnRH antagonist, but in early placenta we found no effect of GnRH. Our finding that hCG did not influence progesterone production in culture, is in accord with that of Paul et al. (42). Unlike steroidogenesis in the ovary, progesterone synthesis by placenta does not appear to be under the control of gonadotropin.

In summary, our present study shows that C_{18} and C_{19} steroids at the doses used have clear-cut effects on progesterone production <u>in vitro</u> and suggests that they may have an important role in the regulation of progesterone synthesis by

the human placenta; however the mechanism of this effect remains unknown.

Table 3.1 Specificity of binding proteins used for assay, calculated at 50% displacement

Steroid	PGP	PAB
Progesterone	100	100
Pregnenolone	2.3	≤ 0.001
5α-androstane-3α,17β-diol	≤ 0.001	0.17
5α-androstane-3β,17β-diol	≤ 0.001	0.36
19-nortestosterone	17	≤ 0.001
androstenedione	2.0	0.1
testosterone	8.0	0.65
5α-dihydrotestosterone	18	0.49
19-norandrostenedione	≤ 0.1	0.15
19-hydroxyandrostenedione	≤ 0.1	0.31
19-hydroxytestosterone	0.4	≤ 0.001
estrone	≤ 0.5	≤ 0.001
estradiol	≤ 0.5	≤ 0.001
estriol	≤ 0.5	≤ 0.001

PGP = Progesterone-binding protein of guinea pig
PAB = Progesterone antibody

Table 3.2 Progesterone values determined before and after chromatography - nmol/L

Treatment*	Before	After
Basal	38	32
	38	38
Androstenedione	464	429
	375	372
5α-androstane-3α,17β-diol	159	223
	260	200
5α-androstane-3β,17β-diol	315	232
	289	264
19-nortestosterone	922	795
	1437	1234

* at 6 μM concentration.

Table 3.3 Summary of effects of C_{18} and C_{19} steroids on estradiol and progesterone production in culture.

Compounds	Estradiol	Progesterone
19-nortestosterone	↑	↑↑↑
Androstenedione	↑↑	↑↑
Androst-5-ene-3β,17β diol	↑↑↑	**NE**
5α-androstane-3α,17β diol	**NE**	↑
5α-androstane-3β,17β diol	**NE**	↑

↑ = Increase production. Number of arrows indicates the degree of stimulation or production
NE = No effect.

Table 3.4 Compounds without effects on progesterone production in culture.

Compound	Concentration
17β-estradiol	1-36 μM
estrone	1-36 μM
estriol	1-36 μM
DHAS	1-36 μM
androst-5-ene-3β,17β diol	1- 6 μM
androst-4-ene-3β,17β diol	1- 6 μM
5β-androstane-3α,17β diol	1- 6 μM
5α-dihydrotestosterone	1-12 μM
testosterone	1- 6 μM
4-hydroxyandrostenedione	1-12 μM
cortisol	1-12 μM
dexamethasone	1-12 μM
monobutyryl-cAMP	1 mM
human chorionic gonadotropin (β-hCG)	20 000 IU/L
gonadotropin-releasing hormone (GNRH	4 μM
Adrenocorticotropin 1-24 (ACTH)	20 μM

Table 3.5 Sulfatase activity in early placenta (conversion of [^{3}H]-DHAS to [^{3}H]-DHA).

Gestational age	% Conversion
6	81
7	73
9	82
9	99

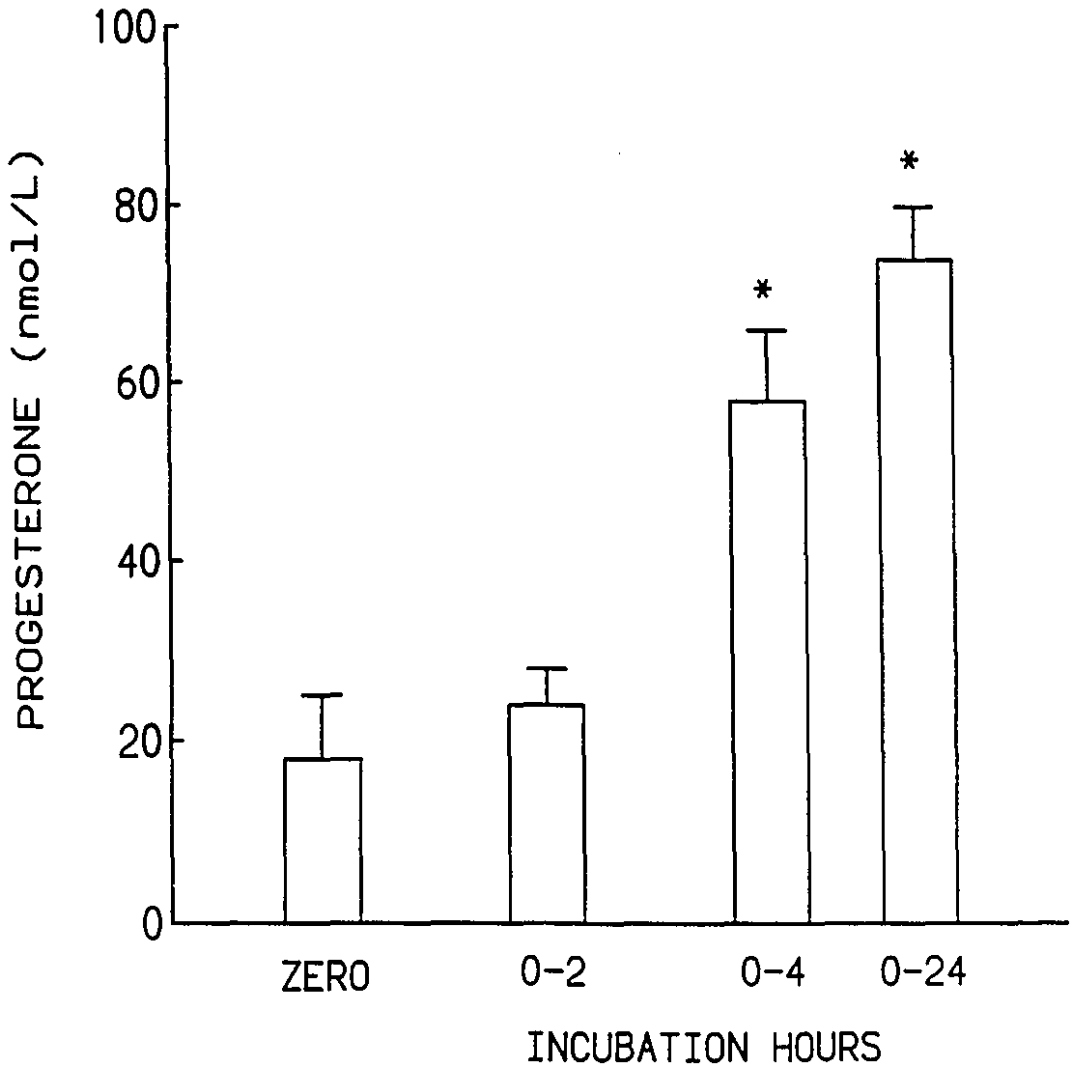

Fig. 3.1 Secretion of progesterone in early gestation placenta in organ culture (Ham's F-10 with FBS and antibiotics) over 24 hours. Each bar represents mean ± SE of duplicate dishes from two different experiments (n = 4). Placental content of progesterone before incubation is indicated at zero time. * indicates significance ($P \leq 0.05$) of difference compared to endogenous concentration at zero time.

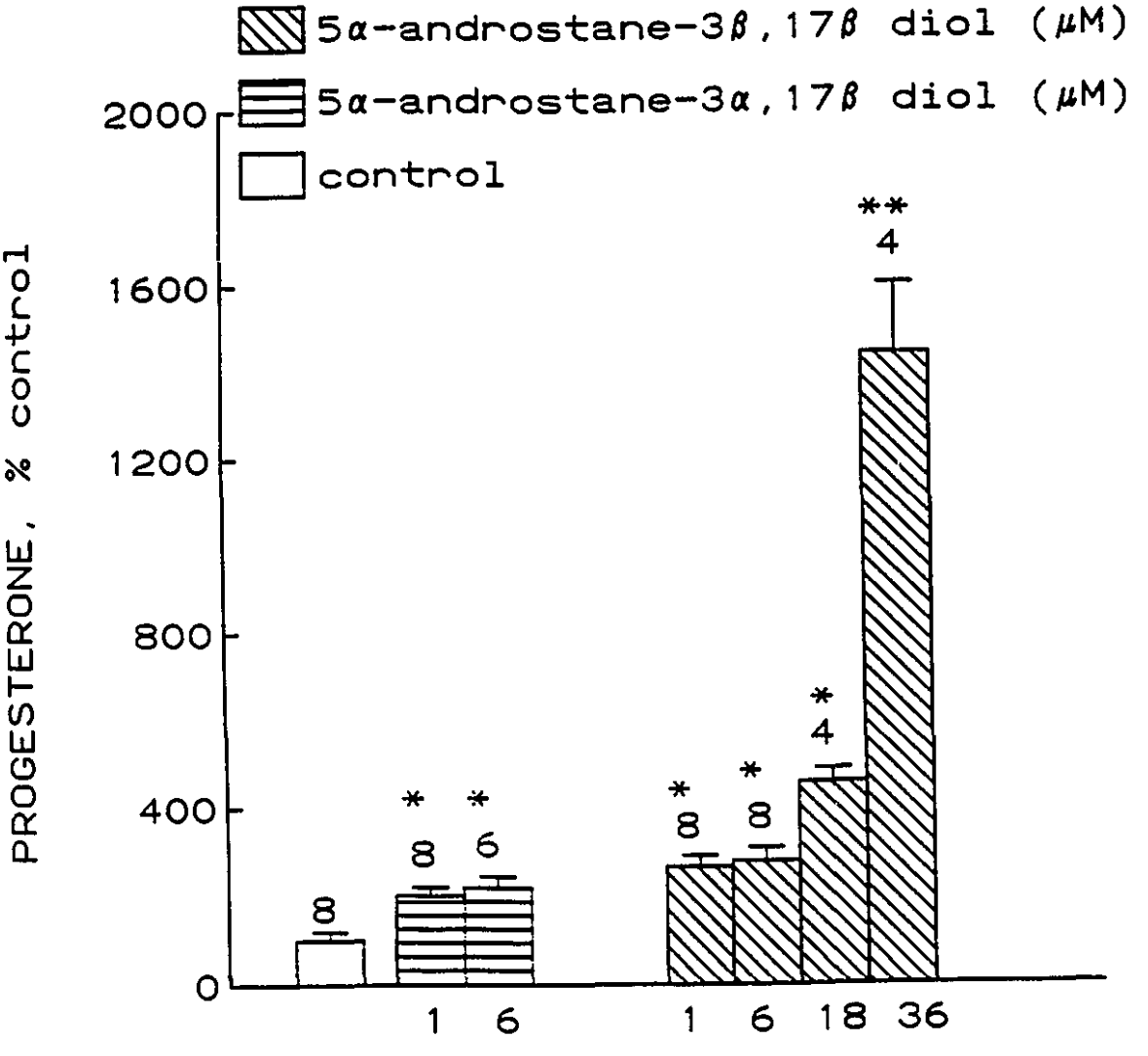

Fig. 3.2 Stimulation of progesterone by 5α-androstane-3ß,17ß-diol and 5α-androstane-3α,17ß-diol in early placental culture. Each bar shows the means ± SE for the number of observations (duplicate or quadruplicate dishes from 2 different placentas). * = $P \leq 0.05$; ** = $P \leq 0.01$.

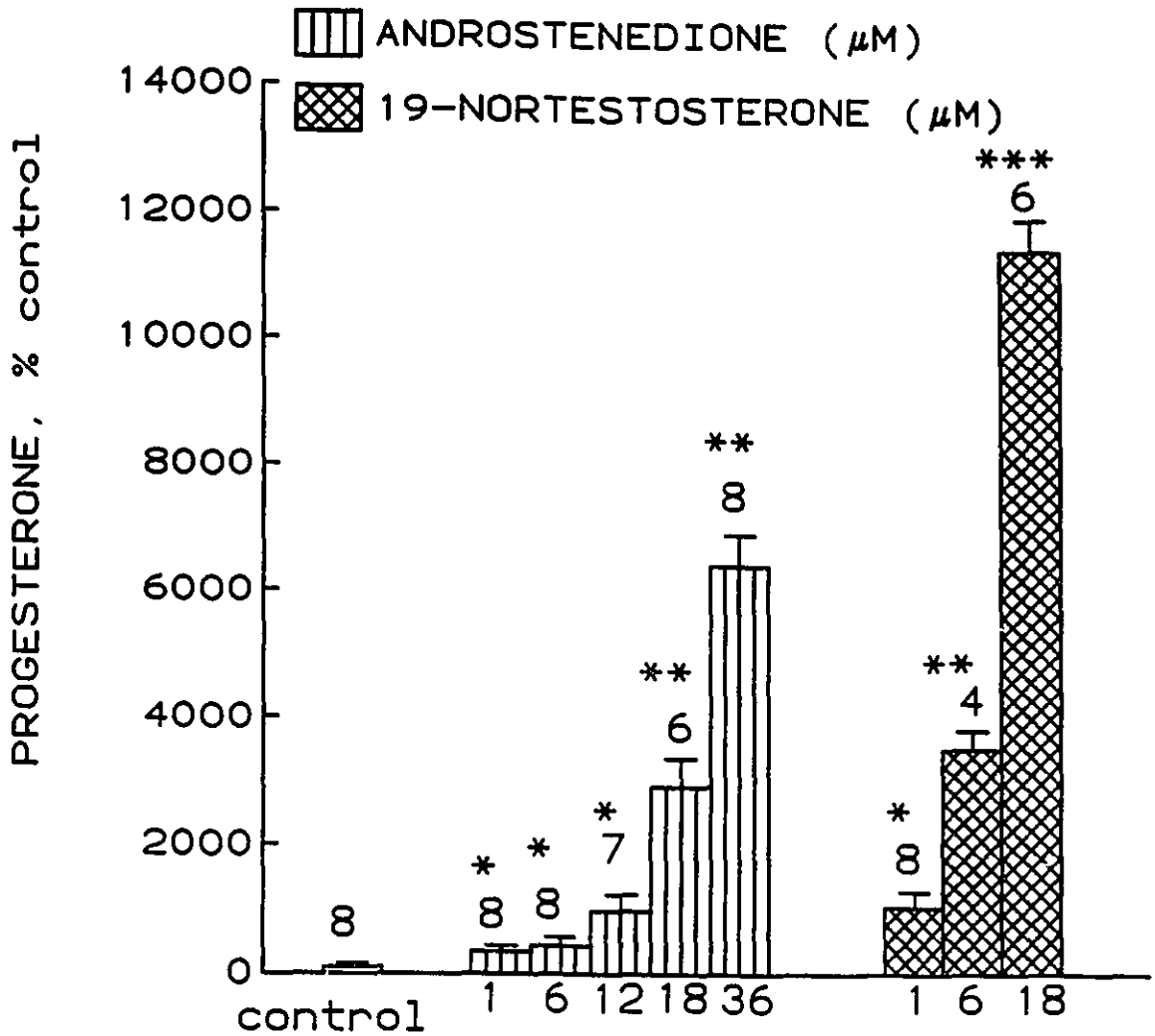

Fig. 3.3 Effect of increasing concentrations of androstenedione and 19-nortestosterone on progesterone production in early placental culture. Each bar indicates the mean ± SE of the number of observations shown at the top of the bar (single or duplicate dishes from 4 different placentas).
* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

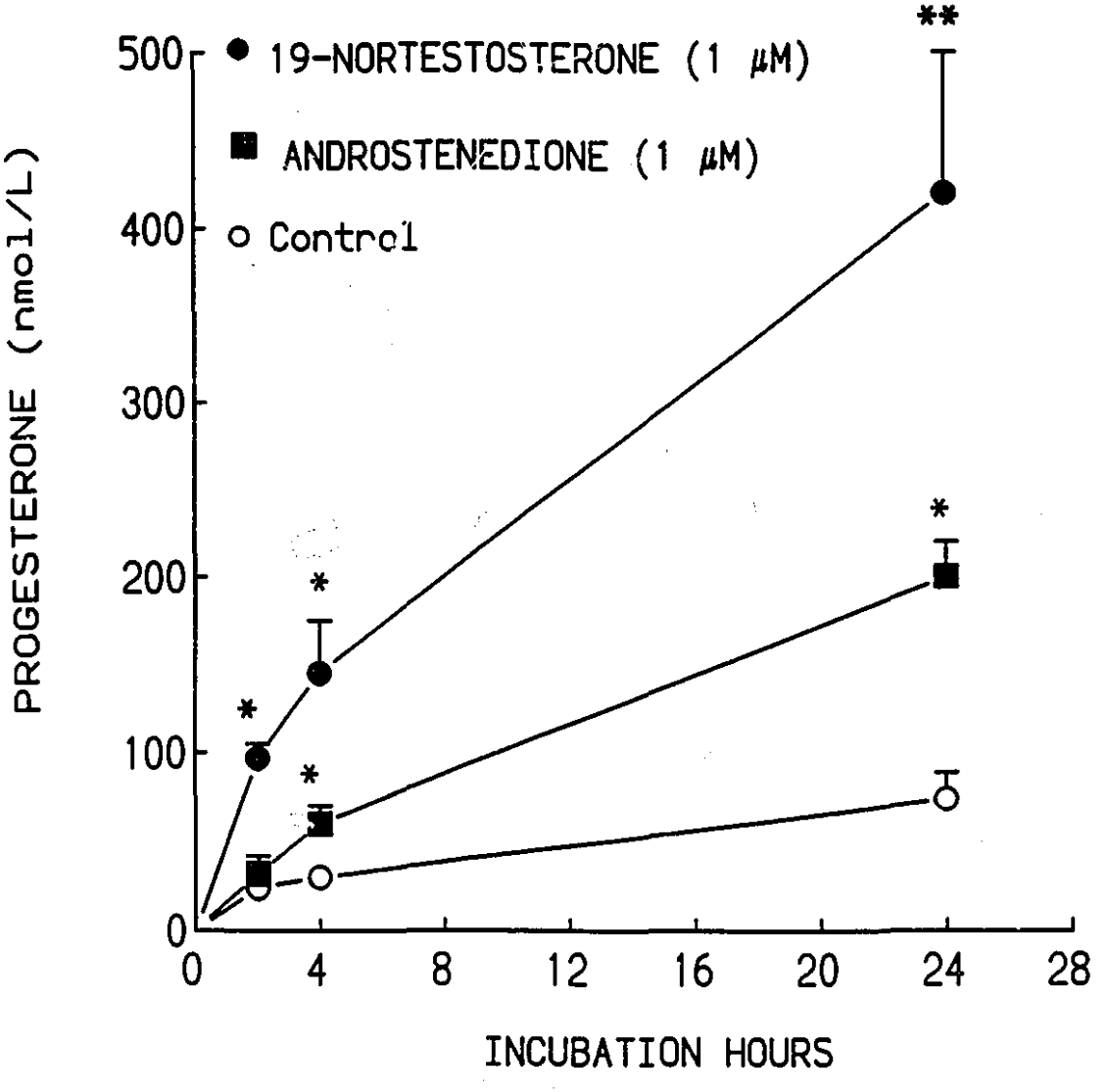

Fig. 3.4 Time course of progesterone stimulation in early placental culture by 19-nortestosterone and androstenedione at a concentration of 1 μM. Cultures were incubated for 2, 4 and 24 hours. Data are the mean ± SE of triplicate dishes from a single placenta. * $p \leq 0.05$; ** $p \leq 0.01$.

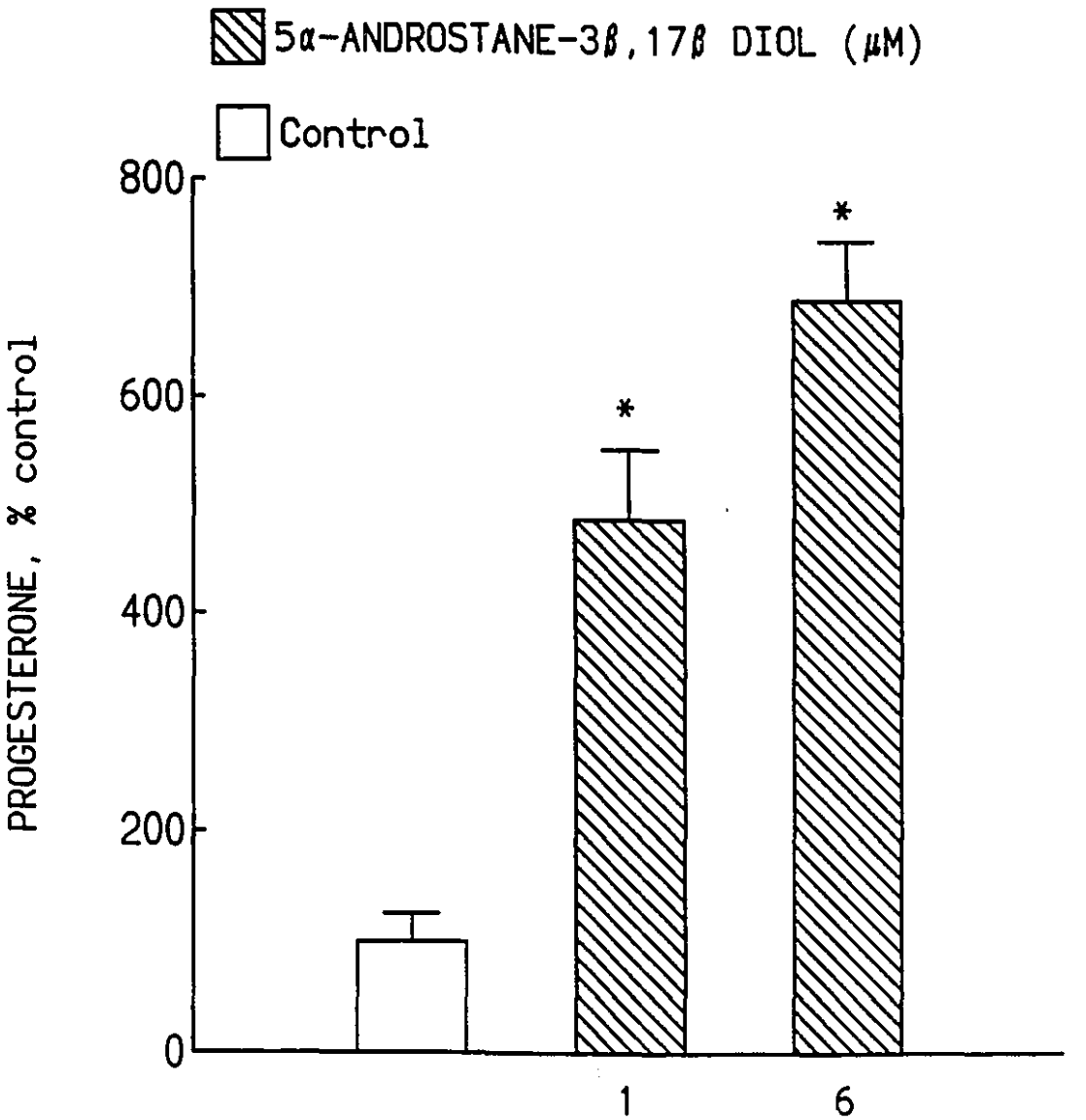

Fig. 3.5 The effect of 5α-androstane-3ß,17ß-diol (3ß-diol) on progesterone production in term placental explants. Each bar indicates the mean ± SE of four observations (duplicate dishes from 2 different placentas). * $p \leq 0.05$.

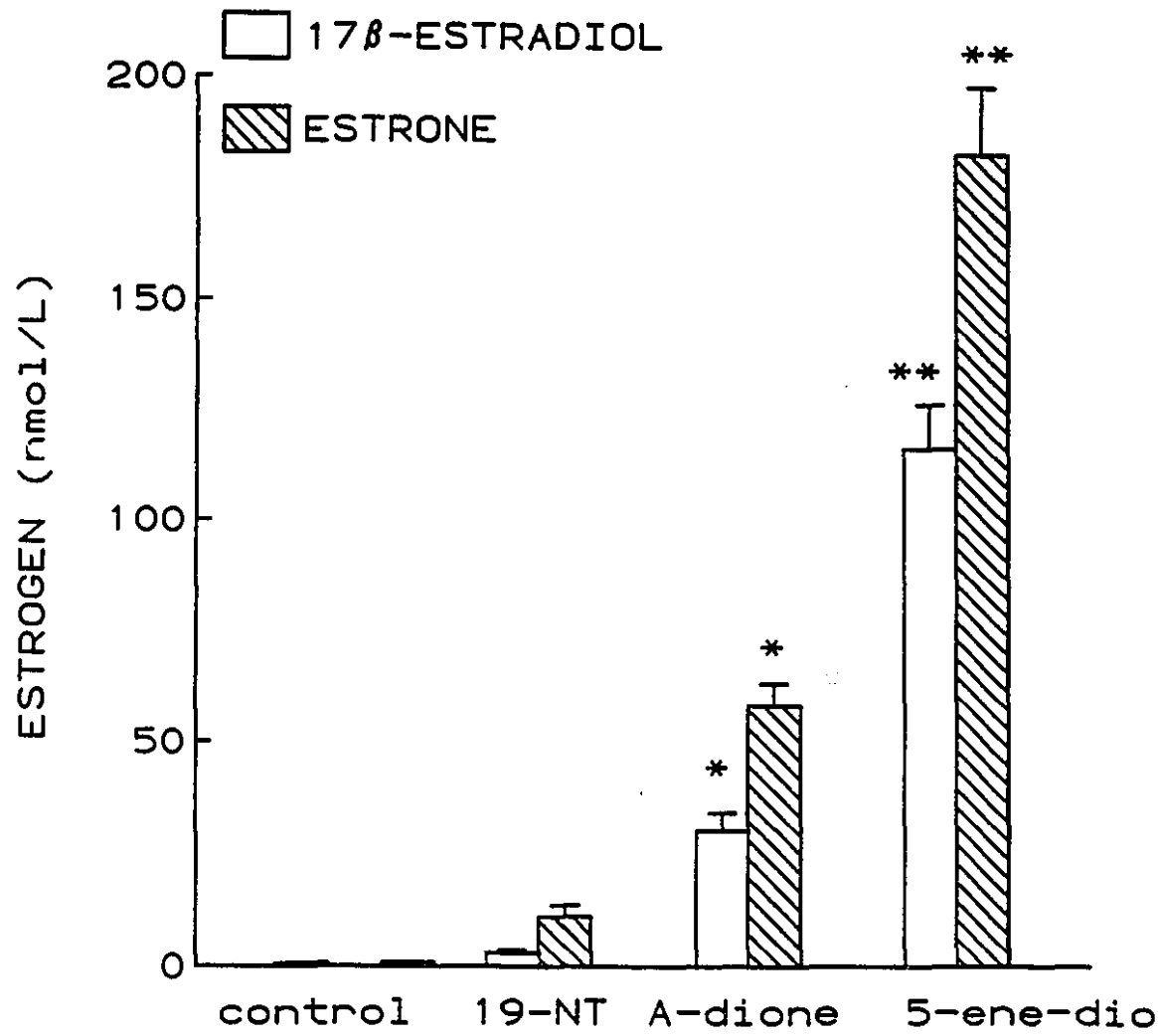

Fig. 3.6 Comparison of aromatization of androstenedione, 19-nortestosterone and androst-5-ene-diol. The results are the mean ± SE of quadruplicate observations (duplicate dishes from 2 different placentas). * $p \leq 0.05$; ** $p \leq 0.01$.

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CHAPTER IV

CONCENTRATIONS OF 19-NORTESTOSTERONE AND SOME ANDROGENS IN HUMAN PLACENTA[1]

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4.1 ABSTRACT

We recently observed that certain steroids stimulate progesterone production in culture in early pregnancy. They include androstenedione (A-dione), 19-nortestosterone (19-NT), 5α-androstane-3α,17ß-diol (3α-diol) and 5α-androstane-3ß,17ß-diol (3ß-diol). To understand the physiological significance of this stimulation, we measured the concentrations of these steroids in pooled placental tissue obtained at different gestational ages. After separation by Sephadex LH-20 column chromatography, elution fractions corresponding to the various steroids as identified by radioactive tracers run in parallel, were assayed by competitive binding to antibodies or transins (human sex hormone-binding globulin, guinea pig progesterone-binding globulin). The results suggested that the concentrations of the more strongly stimulating steroids (A-dione and 19-NT) are higher in the first half of pregnancy compared to term (2- and 10-fold, respectively), while those of the weakly stimulating steroids (3α- and 3ß- diols) rise through pregnancy. These observations are in accord with the concept that stimulation of progesterone production by A-dione and 19-NT is more important, particularly in early pregnancy, while that of 3ß-diol may be significant in later pregnancy.

4.2 INTRODUCTION

It is well known that placenta carries out a wide range of metabolic activities. Although many data are available regarding the steroid concentrations in maternal serum, relatively little attention has been paid to the local tissue concentrations of steroids in placenta, despite their potential physiopathological importance.

Human placenta is supplied with androgen precursors from both fetus and mother (1), but the biological significance of those which are not aromatized to estrogen is unknown. An influence of such steroids on different metabolizing enzymes such as sulphatase (2), 3β-hydroxysteroid dehydrogenase (3β-HSD) (3,4) and aromatase (5), has been reported in placenta. Recently we observed that certain C_{18} and C_{19} steroids (A-dione, 19-NT, 3α- and 3β-diol) stimulated progesterone production in early human placental explant cultures (6). To determine whether this stimulation is physiologically relevant, we measured the placental concentrations of these steroids after chromatography, and for 19-nortestosterone also without chromatography.

4.3 MATERIALS AND METHODS

Samples

Samples of human placenta at different gestations were collected, with the approval of local ethics committees, as

follows: tissues from 6-10 weeks gestation were from healthy women attending a pregnancy termination unit; those at 11-24 weeks were obtained at therapeutic abortion after dilation and curettage or at hysterotomy; those at term (38-40 weeks) were obtained either after elective cesarean sections or normal vaginal delivery. All the specimens were stored at $-80^{0}C$ until processed.

Materials

Radioactive steroids namely [6,7-^{3}H(N)]19-NT, SA 19 Ci/mmol, [1,2,6,7-^{3}H(N)]A-dione, SA 108 Ci/mmol, [1,2,6,7-^{3}H(N)]Testosterone (T), SA 94 Ci/mmol, [1,2-^{3}H(N)]5α-dihydrotestosterone (DHT), SA 52 Ci/mmol, and [1,2,6,7-^{3}H(N)]progesterone (P4), SA 112 Ci/mmol were obtained from New England Nuclear Corporation (Boston, MA). These were diluted with redistilled ethanol to a concentration of 50 μCi/mL and stored at $-10^{0°}C$. The radiochemical purity of the radioactive steroids was checked by Sephadex LH-20 column chromatography using a suitable solvent system. The purity of tracer steroids used in this study was more than 95%.

Anhydrous diethyl ether (analytical grade) was purchased from American Chemical Company (Montreal, Quebec). Other solvents, of analytical grade, were from Fisher Scientific Co. (Montreal, Quebec), as was the scintillation counting fluid (Optiphase). Sephadex LH-20 was from Pharmacia Fine Chemicals (Uppsala, Sweden).

Unlabelled steroids were purchased from Sigma Chemical Company (St. Louis, MO). They were dissolved in redistilled ethanol and stored at -10^0C.

As a source of sex hormone-binding globulin (SHBG), blood was collected in late pregnancy from normal healthy women attending a local obstetric clinic; after centrifugation and separation, the serum was stored at -20^0C until used. For progesterone determination, guinea pig serum in late pregnancy was obtained from the McGill Animal Resources Centre.

An RIA kit for A-dione was purchased from Diagnostic Systems Laboratories, Webster, Texas. Antiserum for 19-NT was obtained from Endocrine Sciences (Tarzana, CA).

Homogenization

Frozen placental samples were allowed to thaw at room temperature and were blotted dry on paper towels; they were then weighed, minced and hand homogenised in aliquots (0.5 gm) in 2-3 mL saline.

Extraction

In glass stoppered tubes, placental homogenates were extracted twice with five volume diethyl ether. Parallel tubes with added radioactive hormones ([^{3}H]-A-dione, [^{3}H]-19-NT, [^{3}H]-T and [^{3}H]-P4) were processed separately to check recovery, which was 80% - 90%. From each trimester (early, mid-term, and term) 4-5 extracts were pooled and the combined

extracts were dried under air. After adding a few drops of ethanol, the pooled extracts were stored at -10^{0}C for subsequent chromatography.

Sephadex LH-20 column chromatography

Five columns (39 x 0.8 cm) were set up and used as described by Murphy (7). Heptane: methylene chloride: ethanol - 50:50:1 was used for elution. Fractions (2 mL) were collected up to 70 mL, after which 5 mL fractions were collected to 160 mL. Each fraction was dried, redissolved in 1 mL ethanol and aliquots were taken for hormone analysis. Radioactive tracers were run in parallel; eluate fractions were dried and counted directly in a liquid scintillation counter after adding 2 mL scintillation fluid. From the patterns of radioactivity, the regions for particular steroids were identified and recoveries from the columns were estimated. For each batch of three samples run together, the 4th column was used for radioactive reference steroids. Elution volumes for the various peaks have been shown to be consistent when run together (7).

Hormone analysis

Fractions corresponding to each tracer peak were assayed by specific radioimmunoassays (for 19-NT and A-dione) or by competitive binding to SHBG (for T, DHT, 3α- and 3ß-diol) (8,9) or the late pregnancy guinea pig progesterone-binding

globulin (for progesterone) (6,10). The specificity was increased by chromatography. The values for each peak were calculated by summing those comprising the peak and correcting for procedural losses. Solvent blanks were negligible.

Although the cross-reaction of progesterone relative to A-dione in the A-dione assay was less than 0.1%, because of the very high concentration of progesterone in placenta (1-2 μg/gm) (3-6 μmol/kg), this interference was not negligible; and since there was partial overlapping of its elution region with that of A-dione, the interference was calculated according to the corresponding values in the progesterone assay, and subtracted from the A-dione values.

Intra-assay variations ranged from 4-8% while those between assays varied from 6-16%.

4.4 RESULTS

A-dione

Placental concentrations of A-dione at different gestations are compared in Fig. 4.1. The mean concentration was twice as high in the early placental pool (30 ng/gm tissue) (100 nmol/kg) as that in the late placental pool (12 ng/gm) (40 nmol/kg). In the mid-gestational pool, the value was intermediate (22 ng/gm) (73 nmol/kg).

19-NT

Figure 4.1 compares the levels of 19-NT in placental pools at different gestational ages. Mean levels in early pregnancy (8.5 ng/gm) (30 nmol/kg) were more than ten-fold higher than those in late gestation (0.5 ng/gm) (2 nmol/kg), while midgestational levels were intermediate (4.5 ng/gm) (16 nmol/kg).

Since the RIA for 19-NT appeared to be highly specific for 19-NT, immunoreactivity was measured in unchromatographed extracts (Fig. 4.2). There was a peak at 7-12 weeks, after which the level declined steadily to 18 weeks with possibly a slight increase at 20 weeks. Very low levels were observed at term. The levels with (Fig. 4.1) and without (Fig. 4.2) chromatography were similar.

3α- and 3ß-diol

When placental eluates from the more polar fractions were assayed by competitive protein binding to SHBG, the regions for the androstanediols (A-diol) and androstenediols

(E-diol) overlapped somewhat, as found by Murphy (8).

Since the α- and ß-diol and androstenediol cross react with testosterone to approximately the same extent (8) and since they eluted close together, their concentrations were measured in testosterone equivalents (TE) as total SHBG bound activity over the diol region. As shown in Fig. 4.3, the mean levels of A-diol + E-diol in placental pools were found to increase with the advancing gestational age (3.2 ng TE/gm in early, 9.7 in mid and 17 in term gestation).

Testosterone (T) and 5α-dihydrotestosterone (DHT)

The mean amount of T was similar in both pools of early (2.7 ng/gm) (9 nmol/kg) and late (3.2 ng/gm) (10 nmol/kg) gestations (Fig. 4.4). The mean level of DHT in the late placental pool (1.5 ng/gm) (5 nmol/kg) was not significantly different from that in the early pool (0.8 ng/gm) (2.7 nmol/kg).

4.5 DISCUSSION

There have been very few studies of the human placental concentrations of androgens or androgen-related steroids, particularly in early gestation. In this study we have measured certain steroids in pools, obtained from early, mid-gestation and term placentas, after chromatographic purification.

It is well recognised that A-dione in the placenta is synthesized from dehydroepiandrosterone of maternal and fetal origin. Although the serum level of A-dione in pregnancy is known to be higher in pregnancy (11,12), we did not find any studies reporting the placental A-dione concentrations. Our observation of A-dione at term (about 12 ng/g) is comparable to the level of its precursor, 19-hydroxyandrostenedione (16 ng/gm) quantitated recently in placenta at the same gestation by gas chromatography-mass spectrometry by Osawa et al (13). Our observatiom of a fall in the level with advancing pregnancy from about 30 ng/g to 12 ng/g is probably due to an increasing capacity for estrogen formation. Siiteri and MacDonald (14) reported that estradiol formation increases from 2% - 4% of intravenously administered DHAS in the first trimester to 35% - 55% at term. Smith and Axelrod (15) also reported increased estrogen synthesis during gestation. The rise in the estradiol level in plasma during late pregnancy (16) has been considered to be due to increasing placental

capacity for the aromatization of androgen with the progress of pregnancy (15). The tissue concentration of estradiol was lower in early gestation and higher at term, supporting the concept of increasing capacity of placental aromatization toward term (17). From these observations it seems reasonable to suppose that A-dione, the known precursor of estrogen, will accumulate in early placenta due to its low capacity for aromatization, and that the level will be lower at term when placental capacity of estrogen biosynthesis increases.

However, the data concerning the effect of gestational age on plasma A-dione level in pregnancy are less clear. No change during pregnancy was found by Harrison et al. (1980) (11) and Martin et al. (18), whereas Nagamani et al. (19) documented higher plasma A-dione levels at 26 - 40 weeks than at 14-20 weeks of gestation. The tissue levels of A-dione we observed (~ 12 - 30 ng/g; 40 - 100 nM) were about ten fold higher than the plasma levels reported in pregnancy (2 - 5 ng/mL) (18,19), suggesting a placental contribution to the plasma A-dione level. The higher level of A-dione in early placenta makes it reasonable to suppose that in early pregnancy it acts physiologically to stimulate placental progesterone production.

We are not aware of any study of the human placental concentration of 19-NT. The specificity of the assay used here, combined with the separation of many other steroids by chromatography, makes it likely that we are measuring only

this steroid. Although the presence of 19-NT in placental tissue has not been documented before, available evidence in the literature has suggested its natural existence in human placenta. 19-NT has been demonstrated as a naturally occurring steroid in man and in animals in biological sources other than placenta. In 1960, Short (20) first reported the presence of 19-norsteroid in mare follicular fluid. Later, 19-NT was identified in human follicular fluid (21) as well as in pregnancy plasma (22). The presence of 19-NT in pregnancy and its absence in the nonpregnant state (22), suggest its origin from the placenta, an organ found only in pregnancy. Milewitch et al. (23) have shown the formation of 19-NT from T during the incubation of baboon term placental microsomes. Since the metabolism of steroids in baboon and human placentas are similar, the natural existence of 19-NT in human placenta as observed by us, was not unexpected. The presence of hydroxylated precursor (19-hydroxyprogesterone) of 19-norsteroid (19-norprogesterone) in human placental extracts (24) provides further evidence pointing to placental synthesis of 19-norsteroids. In the above study (24) of Melby et al, a preliminary finding suggested the presence of another norsteroid, 19-norprogesterone in human placenta. The presence of 19-NT in human placental extracts, as reported here, suggests a biosynthetic pathway exists for the formation of 19-norandrogen in placenta.

Since 19-NT was found to be a poor substrate for placental

aromatization (6,25,26), its biosynthesis may follow a path other than aromatization. Osawa and Yarborough (27,28) recently discovered a non-aromatizing androgen 19-desmolase enzyme activity which produces 19-norandrogen in the dog adrenal. The existence of a similar enzyme system in human placenta for the formation of 19-norsteroids, seems likely. Recently, a newly discovered 19-norsteroid, estradienolone, was shown to have higher placental level at mid-gestation than at term (29,30).

On the basis of all the above observations, it seems likely that 19-NT in the human placenta as observed by us, is physiologically relevant. The concentrations of placental 19-NT measured here (0.5 - 8.5 ng/g; 2-30 nM) are about 100 times greater than those reported in pregnancy serum (20-60 pg/mL) (22). The higher levels of 19-NT in early pregnancy as observed in this study, appear to correlate with our previous observations of the stimulation of progesterone synthesis by 19-NT in early pregnancy (6), and may be important with respect to the luteo-placental shift. In contrast to our finding of decreasing levels in placenta with gestational age, 19-NT was found to increase in plasma through pregnancy as found by Reznik et al. (22). Presumably, this difference can be accounted for by the increasing mass of the placenta with the advance of gestation.

At present we do not have any information available regarding in situ synthesis of 5α-reduced metabolites of T in

human placenta. The isolation of 5α-reduced metabolites from the human placental incubation of radiolabelled dehydroepiandrosterone (15) supports our observation, indicating the presence of placental 5α-reductase activity. Although placental concentrations of A-diol and E-diol, have not been reported previously, a significant elevation of these compounds, has been documented in late pregnancy plasma (8,31,32). The presence of these androgen metabolites in pregnancy plasma strongly supports their presence in placental tissue. In accord with the above concept, the 5α-reduced metabolite of progesterone in plasma has been shown to rise through pregnancy (33,34). Recently, the capacity of human placenta to synthesize the 5α-reduced metabolite, from in vitro incubation of progesterone, has been shown (35). The occurrence of 5α-dihydroprogesterone (DHP) in human placenta has also been reported recently (29,30). The presence of 5α-DHT in placenta as shown in this study (see below) suggests the presence of reductase activity for androgen as well.

The amount of A-diol in different androgen-dependent tissues from postmortem samples of men obtained within 24 hours of death varied from 0.3 - 4 ng/gm) (36). Our value for A-diol and E-diol together (about 7 ng/gm) in the late placental pool is about two-fold higher than that reported for late pregnancy plasma (about 3 ng/mL) (8). Therefore, placenta may make a modest contribution to the elevated diol concentration of serum in pregnancy.

The biological relevance of the rise of A-diol concentration in maternal plasma in late pregnancy, is not known. Our observation of the stimulation of progesterone production by 3ß-diol in late placental culture (6), suggests that 5α-reduced compounds are not merely inactive metabolites of androgen. In accord with this concept, Eckstein et al. (37,38) recently reported a regulatory role for 3ß-diol in the secretion of luteinizing hormone in the immature female rat. Also, the binding of 3ß-diol with a specific protein, presumably an estrogen receptor, in male rat pituitary (39) and its induction of some nuclear activities similar to that of estradiol (40), have been documented recently.

The importance of other 5α-reduced steroids has also been documented. Grimshaw et al. (41) reported the stimulatory effects of 5α-dihydroprogesterone on progesterone production in term placental culture, while the study of Everette et al. (42) suggested the importance of 5α-dihydroprogesterone in the maintenance of refractoriness to the pressor effect of angiotensin II during human pregnancy. All these studies support the physiological significance of 5α-reduced steroids in the human placenta. Human placental DHT has not been measured previously. However the <u>in vitro</u> influence of DHT on placental estrogen (43,44) as well as on progesterone (41), has been reported. The presence of DHT in the human placenta reported here suggests that the above observations have some physiological importance. Since the amount of DHT in serum is

high in pregnancy compared to the nonpregnant state (11,19), it is reasonable to assume the availability of DHT in placental tissue as observed by us. Placental DHT levels (0.5 - 1.7 ng/gm) were similar to the plasma levels observed in pregnancy (0.04 - 2.0 ng/mL) (19). Levels in some androgen-dependent tissues (scrotum, thigh, pubis, etc.) from men (0.3-1.5 ng/gm) (41) were also similar.

Placental concentrations of T have not previously been measured. Inhibition of β-hCG production by T was reported in early human placental culture (45). Harrison et al. (11) have reported that there was no variation of maternal plasma T with gestational age. Our placental T levels (2.7 - 3.2 ng/gm) also did not vary through pregnancy and were similar to those found for androgen-dependent tissues in men (0.4 - 2 ng/gm) (36).

In our previous study (6) these steroids were effective in stimulating progesterone production at 1 μM concentrations, 10-100 fold higher than the concentrations found here. However it is well recognized that <u>in vitro</u> effects often require higher concentrations than <u>in vivo</u>. Thus it seems likely that the concentrations of ~10 ng/gm of 19-NT and of 30 ng/g androstenedione, made within the placenta itself, are physiologically relevant; the roles of T, DHT and the diols are less convincing.

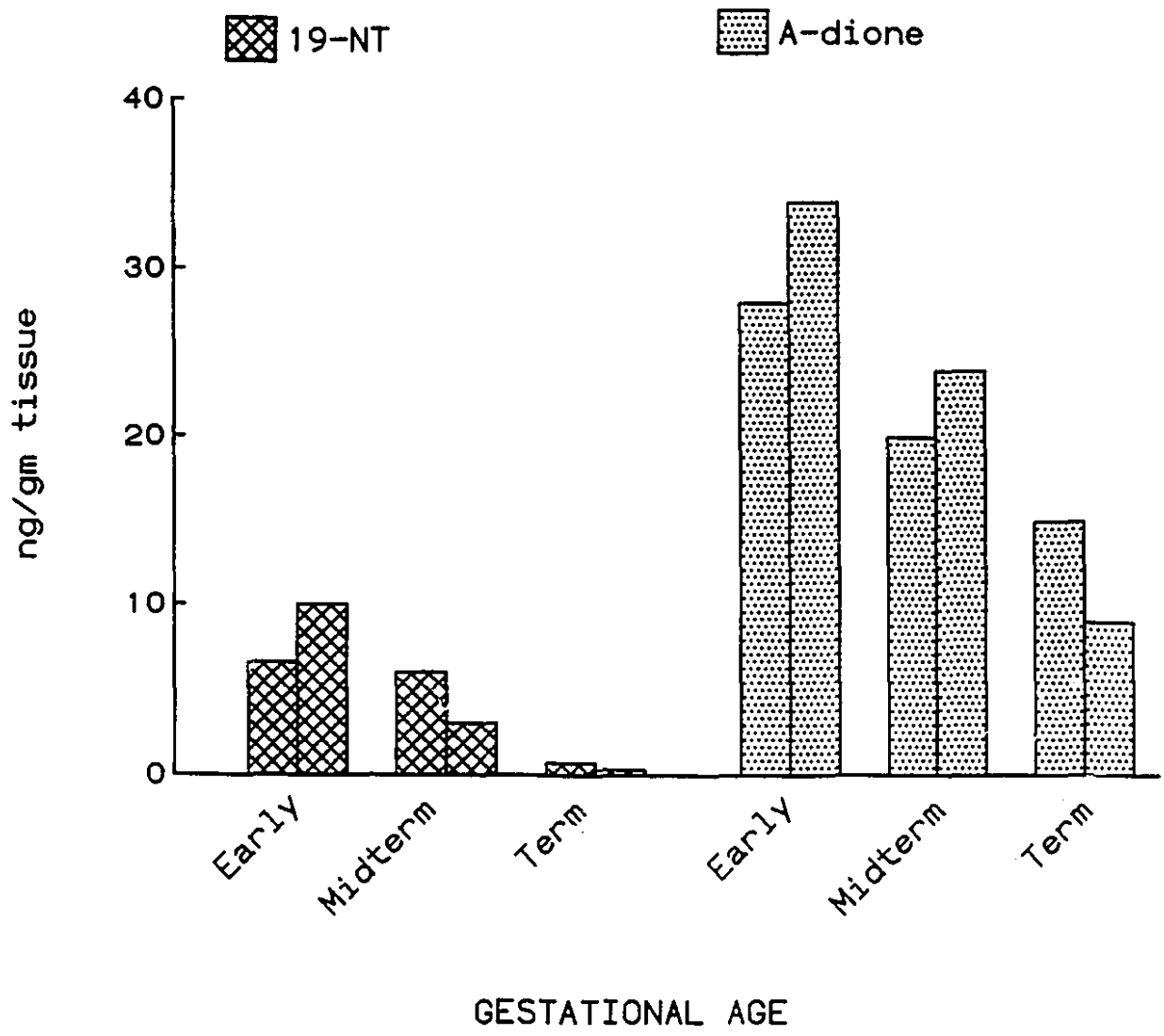

Fig. 4.1 Concentrations after chromatography of A-dione and 19-NT in placental pools are compared at different gestational ages. Each bar indicates one value for a pool of 4-5 samples. Values are corrected for procedural losses.

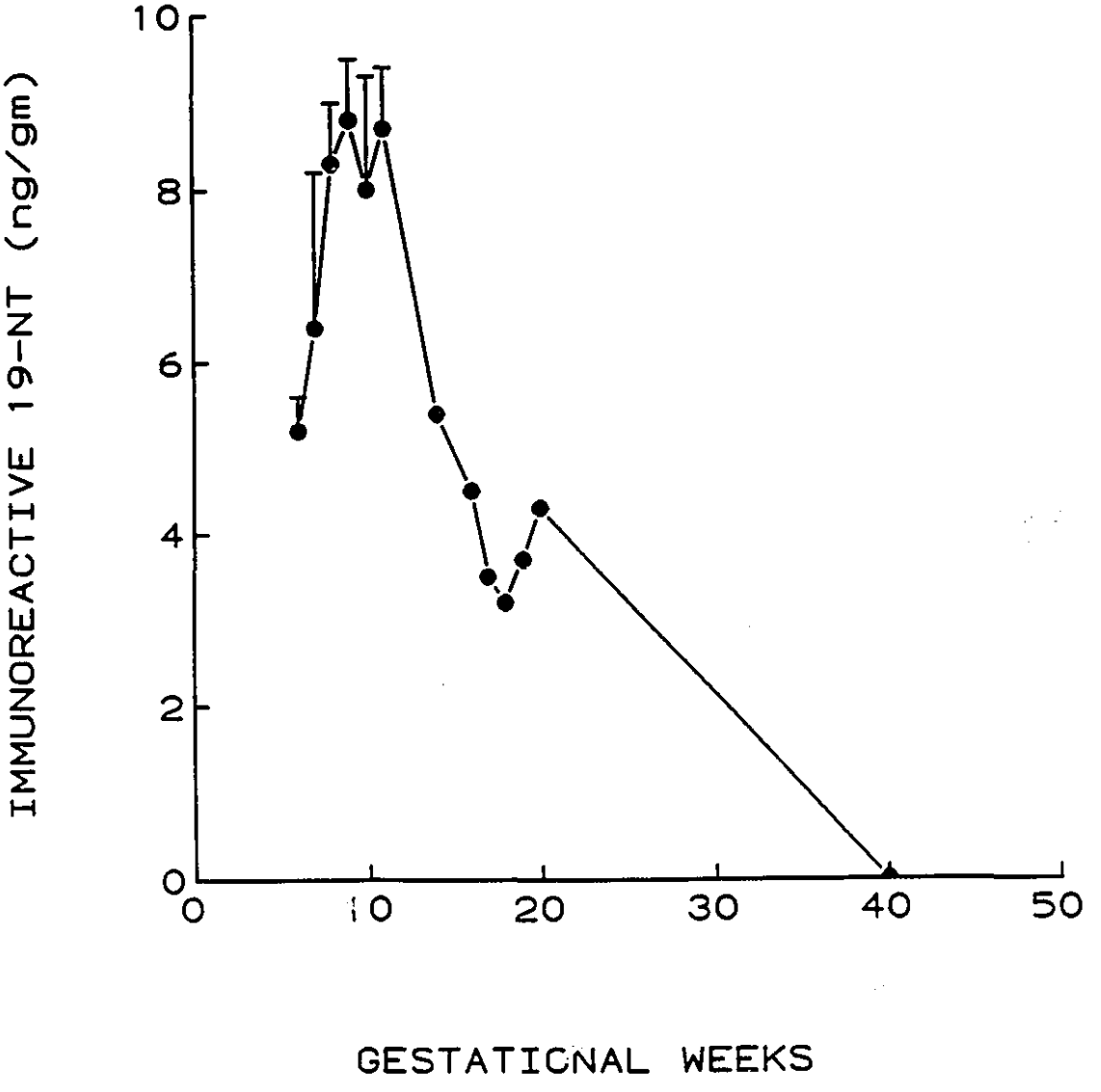

Fig. 4.2 Immunoreactivity of 19-NT in unchromatographed samples of placental tissue through gestations. Data points with error bars indicate mean ± SE for values from four different placentas determined in duplicate; other points indicate single samples determined in duplicate. The levels in 4 term placentas were all very low, so that the error bar does not show up on the graph.

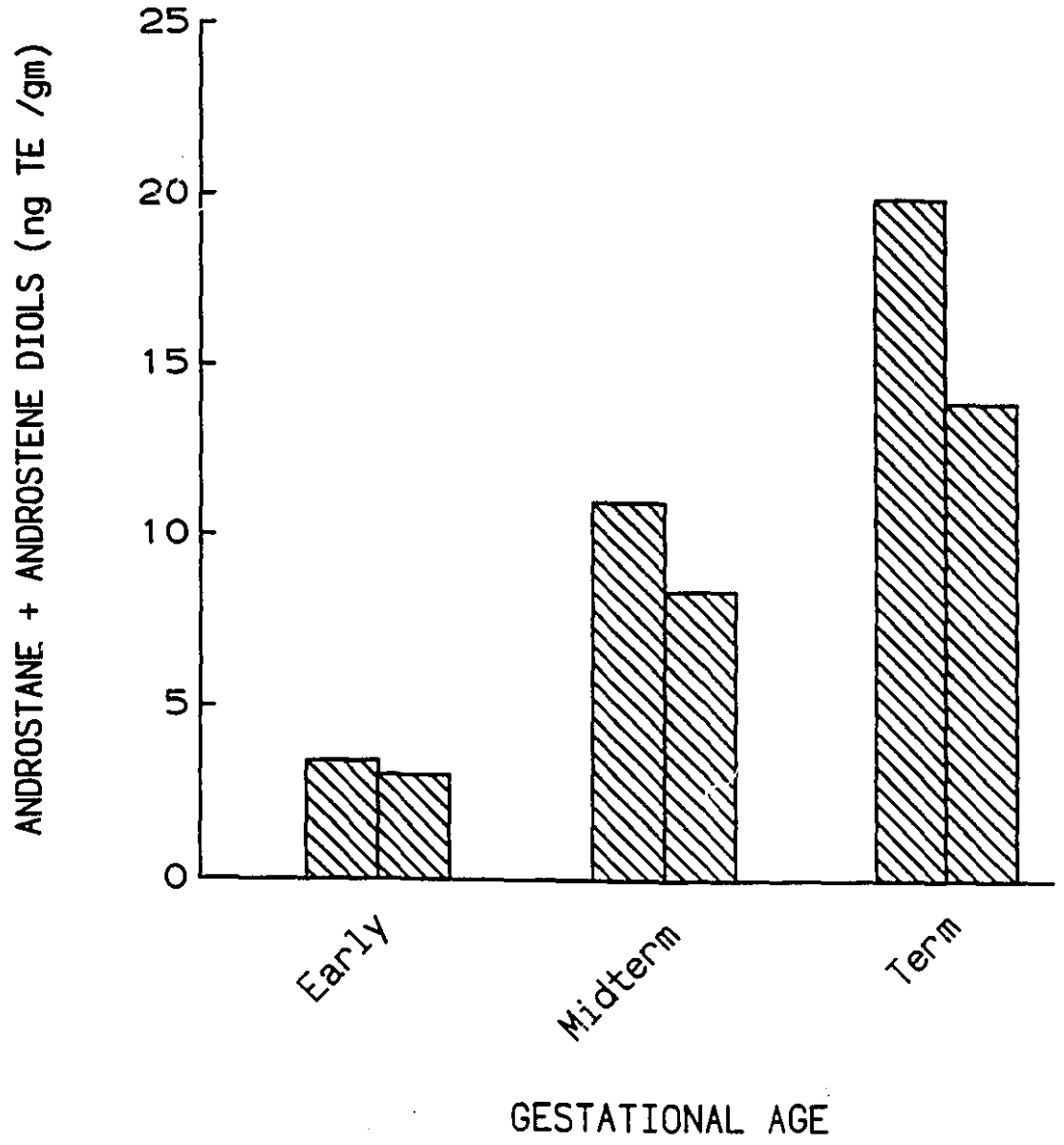

Fig. 4.3 Placental levels of androstanediol (A-diol) plus androstenediol (E-diol) measured in testosterone equivalent (TE) determined as the total SHBG-bound activity over the diol region, are compared at different gestational ages. Each bar indicates one value for a pool of 4-5 samples. Values are not corrected for procedural losses.

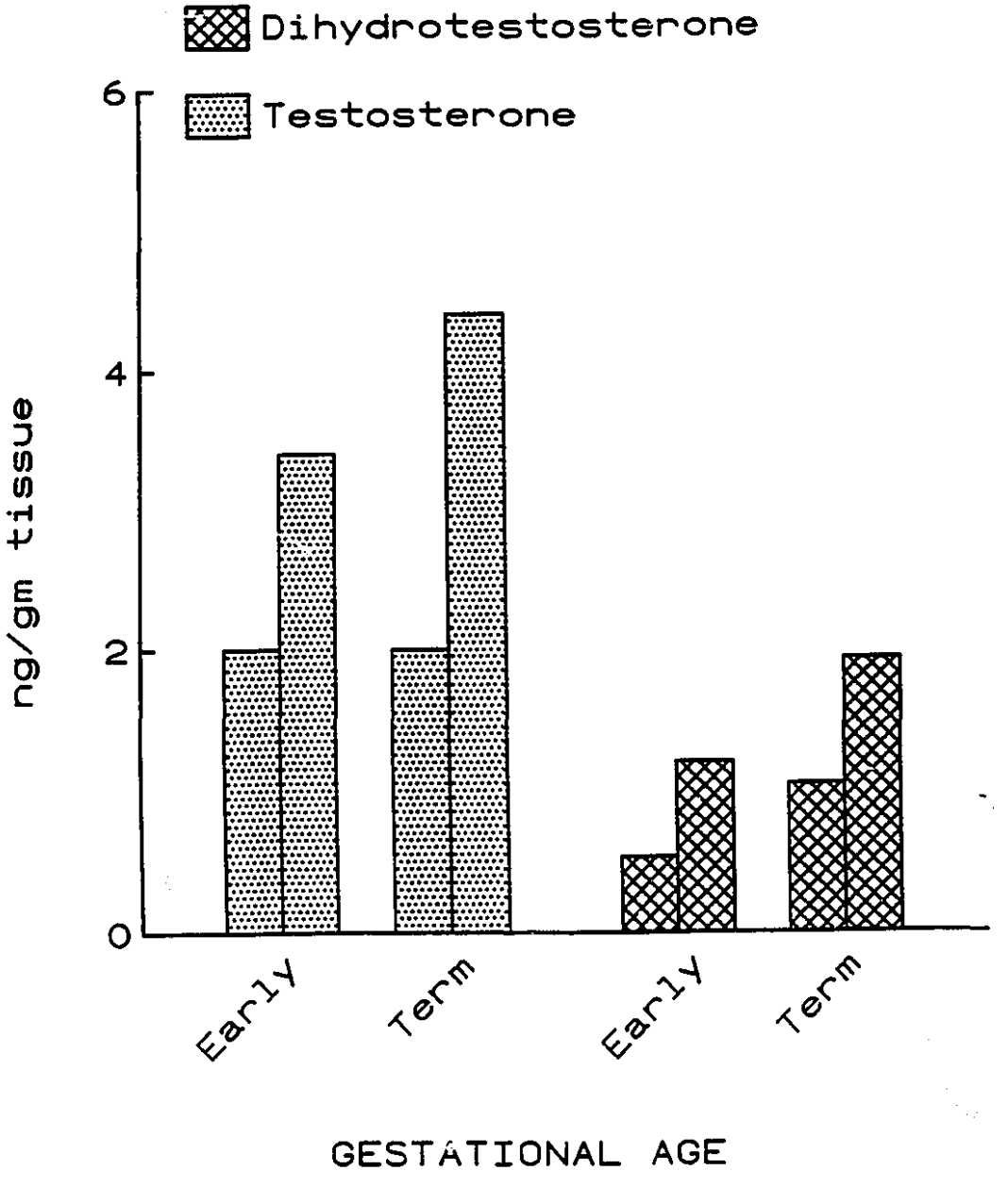

Fig. 4.4 The levels of testosterone and dihydrotestosterone in placental pools from early and term gestation. Duplicate observations are shown. Each bar indicates one value for a pool of 4-5 samples. Values are corrected for procedural losses.

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CHAPTER V

INFLUENCE OF 19-NORTESTOSTERONE AND ANDROGENS ON PROGESTERONE BIOSYNTHETIC ENZYMES IN EARLY HUMAN PLACENTAL EXPLANTS[1]

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Short title: Regulation of progesterone biosynthesis

Key words: early human placenta, progesterone, biosynthetic enzymes, androgens, 19-nortestosterone, androstenedione

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5.1 ABSTRACT

We recently showed that the production of progesterone (P4) in human placental explant culture from early gestation is enhanced by treatment with 19-nortestosterone (19-NT) or with certain androgens, namely androstenedione (A-dione), 5α-androstane-3α,17β diol (3α-diol) and 5α-androstane-3β,17β diol (3β-diol). This stimulation of P4 was explored further in this study. There was little metabolism of radioactive P4 when incubated for 24 hours in the presence or absence of these steroids.

The role of different steroids in the regulation of P450 cholesterol side chain cleavage enzyme (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD) was evaluated by measuring the conversion of P4 derived from unlabelled 25-hydroxycholesterol (25-OHC) and from labelled pregnenolone, respectively. The results showed that 19-NT, A-dione and 3α-diol stimulated P450scc activity; however, 3β-diol was ineffective. While 19-NT and 3β-diol enhanced the bioconversion of pregnenolone to P4, A-dione and 3α-diol were without effect.

The initial rapid stimulation of P4 by 19-NT within two hours of incubation was not blocked by concurrent treatment with cycloheximide (CH). However, after incubation for 24 hours, 70% of the 19-NT- stimulated P4 was abolished by CH. During the same incubation period, P4 stimulation by A-dione,

3α- and 3β-diol were completely blocked by treatment with CH. Thus our observations suggest that 19-NT-stimulated P4 accumulation is due to the combined effects on P450scc and 3β-HSD enzyme activities. A-dione and 3α-diol increase biosynthesis of P4 by acting selectively on P450scc enzyme. However, the stimulatory action of 3β-diol on P4 is only at the level of 3β-HSD. Since CH blocks the stimulatory actions, the mechanism(s) by which androgens (A-dione, 3α-diol and 3β-diol) and norandrogen (19-NT) augment the biosynthetic enzyme activities appears to be mediated by a process inhibited by CH. Since CH interference was absent during the initial rapid P4 stimulation by 19-NT, there may be a direct action of this steroid at the cellular level which is not dependent on new protein synthesis.

5.2 INTRODUCTION

After 7-8 weeks of gestation, human placenta is the main source of progesterone (P4) which plays an important role in the maintenance of pregnancy (1). While the biosynthetic pathway of P4 formation from cholesterol via pregnenolone (P5) is well established, there is little known about its regulation. We recently observed that 19-nortestosterone (19-NT), androstenedione (A-dione), 5α-androstane-3β,17β diol (3β-diol) and 5α-androstane-3α,17β diol (3α-diol) stimulate early placental P4 production; 19-NT was the most potent (2). For a better understanding of these stimulatory effects, we investigated the role of these steroids on P4 metabolism as well as on biosynthetic enzymes.

The P450 cholesterol side chain cleavage (P450scc) enzyme - a complex of mitochondrial enzymes containing cytochrome P450 (3), catalyses the conversion of cholesterol to yield the C_{21} steroid P5. 3β-hydroxy-5-ene-steroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD) subsequently transforms P5 into P4, an essential step for the biosynthesis of P4 (4). In addition, the effect of cycloheximide (CH) was investigated to know whether P4 stimulation by the above mentioned steroids requires new protein synthesis.

5.3 MATERIALS AND METHODS

Placental samples (7-10 weeks) were obtained, with their informed consent, from patients undergoing elective abortion. Samples were collected in saline at the time of dilation and evacuation, performed in the Pregnancy Termination Unit of the Montreal General Hospital.

Powdered Ham's F-10 culture medium and fetal bovine serum were purchased from Grand Island Biological Company (Burlington, Ontario). Amphotericin B was obtained from Squibb (Montreal), penicillin G from Glaxo Laboratories (Montreal) and gentamicin sulfate from Schering (Pointe Claire, Quebec). Falcon plastics (Los Angeles, CA) supplied the plastic culture dishes (60 x 15 x 2 mm). Stainless steel grids (2.5 x 2 cm) were from Johnson wire works (Montreal, Quebec). The glass-redistilled (analytical grade) diethyl ether was purchased from American Chemical Company (Montreal, Quebec). The scintillation counting fluid (Optiphase) and other solvents used were from Fisher Scientific Company (Montreal, Quebec). Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Unlabelled steroids, including 25-hydroxycholesterol (25-OHC), were obtained from Sigma Chemical Company (St. Louis, MO), dissolved in redistilled ethanol and stored at -10^0 C. Cycloheximide (Actidione) was from Fluka Chemical Company (New York, NY). Radioactive steroids namely [7-^{3}H(N)]pregnenolone

(SA 17 Ci/mmol), [1,2 $^{3}H(N)$]17α-hydroxyprogesterone (SA 57 Ci/mmol) and [1,2,6,7 - $^{3}H(N)$]progesterone (SA 112 Ci/mmol) were obtained from New England Nuclear Corporation (Boston, MA); these were diluted with ethanol to a concentration of 50 μCi/mL and stored at -10^{0} C.

Placental Explant Culture

Tissues were kept on ice until processed within one to two hours of removal. After rinsing several times with cold saline, placental villi were dissected with sharp scissors and scalpel. Ten explants, about 1 mm^3 each - five on each of two supporting grids - were placed in a culture dish containing 3 mL of control medium composed of Ham's F-10 + fetal bovine serum 10% + antibiotics (penicillin G: 200 IU/mL; amphotericin B: 5 μg/mL; gentamicin sulfate: 40 μg/mL). Thus each culture dish contained approximately 10 mg tissue. Incubation was carried out at 37^{0}C in a humidified environment of 5% CO_2 in air. Media were collected and stored at -20°C until processed.

Extraction of media

Aliquots were extracted once with 5 volumes of diethyl ether, followed by two washes with the same solvent.

Chromatography

Aliquots of extracts were chromatographed on Sephadex LH-20 columns using the solvent system: heptane:methylene

chloride:ethanol 50:50:1 as described by Murphy (5). Aliquots of the eluate fractions were counted in a liquid scintillation counter to locate the radioactivity and calculate recovery. P4 and its metabolites were identified according to the elution positions of the reference steroids (P4, 17-hydroxyP4, 20α-dihydroP4) as previously described (5).

Hormone assays

The P4 concentrations of the extracts were estimated by competitive protein binding (6,7) to pregnant guinea pig serum, which contains a protein with high affinity and specificity for P4 (7,8). Briefly, 0.1 mL protein-tracer solution [late pregnancy guinea pig serum or plasma 10 μL; tracer P4 130 μL (50 μCi/mL); 1.0 mL 0.075M phosphate buffer, pH 7.5; 9.0 mL gelatin water (0.5 gm/L)] was added in duplicate to tubes of each extract and in standard tubes containing 0, 0.2, 0.4, 0.8, 1.2, 2.4, and 4.8 ng P4. The rack containing all the tubes was incubated at 45°C for 5 min, then at 4^0 C for 90 min. While still in the cold bath, 1.0 mL charcoal solution [0.25 gm/L Norit A charcoal, 0.25 gm/L dextran] was added to each tube, the tubes were left standing in the bath for 4 min, then centrifuged at 4^0 C for 5 min. Supernatant 0.5 mL was transferred to a counting vial, and 2 mL Optiphase added. The tubes were shaken and counted to 10,000 cpm. The time in min/10,000 cpm was plotted against the progesterone in ng, and the values of the unknowns were read off the curve.

Along with samples, media without tissue were used as additional controls in each experiment. The P4 concentration of medium without tissue was subtracted from that obtained in the presence of placental tissue. Samples from a given experiment were assayed simultaneously to avoid inter-assay variation, which was about 15%, for progesterone assays. Intra-assay variations were 9% or less. The concentrations of P4 in our study were expressed as ng/dish/day (10 mg tissue) unless otherwise indicated.

P4 metabolism

After allowing the explants to equilibrate for 24 hour, [^{3}H]-P4 (5000 cpm/mL) was added to each dish. Duplicate dishes were incubated for another 24 hour in the presence or absence of 1 μM unlabelled steroids (19-NT, A-dione, 3α- or 3β-diol). Media were collected, extracted and chromatographed, and the recoveries calculated.

P450scc enzyme activity

Preliminary experiments were carried out to validate the increase in P4 production from exogenous 25-OHC added in a range of 0-100 μM in culture dishes incubated for 24 hr. After initial incubation for 24 hours, cultures containing 50 μM of 25-OHC were treated with either of 19-NT, A-dione, 3α- or 3β-diol at 1 μM concentration for a further 24 hr, after which media were collected and analyzed for P4 content.

3β-HSD activity

Incubation was carried out for 24 hrs with tritiated P5 (5000 cpm/mL) in the presence or absence of added steroids. The steroids, 19-NT, A-dione, 3α- or 3β-diol, were added at a concentration range of 1-12 μM. At the end of incubation, media were collected and the steroids were extracted and chromatographed. Recovery was 85% - 90%. The percentage of bioconversion of P5 to P4 was calculated in the presence or absence of steroid treatment.

Cycloheximide (CH) treatment

In initial experiments, culture dishes treated with 19-NT (1 μM) were incubated for fixed intervals of 15 min, 30 min, 2 hours and 8 hours in the presence or absence of cycloheximide (1 mM). In other experiments, the cultures were treated separately for 24 hrs with 19-NT, A-dione, 3α- or 3β-diol either in the presence or in absence of cycloheximide (1 mM). Media were collected at intervals; aliquots were extracted and analyzed for P4 content.

Statistical analysis

From the observations of the replicate experiments, means and standard errors (SE) were calculated. The significance of the difference between control and experiment was determined using Student's t-test. The level of probability accepted as statistically significant was $P < 0.05$.

5.4 RESULTS

Metabolism of P4

There was no significant metabolism of P4 in the control cultures nor in those in which 19-NT, A-dione, 3α- and 3β-diol were added. Almost all of the radioactivity (97 ± 7%, n = 10) was recovered in the P4 region. No significant radioactivity was found in the elution volume of the main metabolite 20α-dihydroP4 (20α-DHP). It was concluded that there was little or no metabolism of P4 under these culture conditions.

Basal conversion of 25-OHC to P4

The basal conversion of P4 from exogenous substrate-25-OHC is shown in Fig 5.1. The P4 production (ng/dish/day or nM, mean ± SE, n = 4) did not increase above the level obtained from endogenous precursor (32 ± 11 ng) until the amount of exogenously added 25-OHC in culture was 50 μM or greater. At 50 μM, the level of P4 rose to 124 ± 20 and at 100 μM to 342 ± 90 ng. The substrate concentration of 50 μM was selected for subsequent experiments with 25-OHC since it ensures a significant amount ($P < 0.05$) of P4 production compared with the endogenous level.

Effects of added steroids on conversion of 25-OHC to P4

To ascertain the effect on the conversion of P4 from exogenous cholesterol, placental explants were treated with 1 μM 19-NT in the presence of 25-OHC. It was found that the P4

production (ng/dish/day or nM, mean ± SE, n = 4) increased about three-fold (578 ± 40; $P \leq 0.01$) over the level found in control culture with 25-OHC in the absence of any treatment (176 ± 34) (Fig. 5.2). Similarly, the separate treatments of cultures containing 25-OHC (50 μM) with 1 μM of A-dione and 3α-diol, respectively, caused an approximately two-fold increase of P4 production in culture (350 ± 55, and 328 ± 80; $P \leq 0.05$, respectively) for each sample compared to control. In contrast, 3β-diol at the same concentration did not augment the conversion of P4 from 25-OHC, the value (166 ± 33) being close to that of control ($P \geq 0.05$).

Effects of added steroids on conversion of P5 to P4

To examine further the influence of these steroids on the conversion of P5 to P4, [^{3}H]-P5 was incubated for 24 hours in presence or absence of steroids (Table 5.1). Without any treatment, the basal conversion of P5 to P4 in our control culture in medium containing [^{3}H]-P5 was minimal (4.3% ± 2, n = 3). However, when the placental explants were treated with 19-NT (1, 6, 12 μM) the bioconversion was enhanced (2-7 fold) in a dose dependent fashion (8, 16 and 29%, respectively). Likewise, 3β-diol at a dose range of 6 and 12 μM increased the conversion rate to 12% and 20%, respectively. Parallel observations with A-dione and 3α-diol at the dose range of 1-6 μM did not show any influence on the conversion of [^{3}H]-P5 to [^{3}H]-P4.

Effects of cycloheximide on the stimulation of P4 production

The results of the short-term incubation (15 min to 8 hours) study with 19-NT (1 μM) in the presence or absence of CH treatment (1 mM) is shown in Fig. 5.3. With increasing time, there was a gradual increase of mean basal progesterone production from 22 to 84 ng/dish/day or nM. Treatment with CH did not alter the pattern of basal P4 production within this interval. After 2 to 8 hours of incubation in the presence of 19-NT, there was increased accumulation of P4 in the medium (from 225 to 633 ng/dish or nM). At all the time periods, concurrent addition of CH did not affect 19-NT-stimulated P4 production significantly ($P \geq 0.05$).

Experiments were also done to observe the effects of CH in cultures incubated for a longer period of 24 hours (Fig. 5.4). In control dishes mean P4 production was 80 ± 20 ng/dish (nM) while CH treatment caused about 50% reduction of basal P4 production (to 32 ± 20). 19-NT (1 μM) stimulated P4 production about seven-fold (to 560 ± 183 ng/dish or nM) compared to control. However, in the presence of CH, about 70% of this stimulatory action was lost (166 ± 92 ng/dish or nM). A-dione (1 μM) stimulated P4 production about four-fold (to 358 ± 40 ng/dish/day or nM) compared to control, an effect which was abolished by CH. Similarly, in the absence of CH, 3β-diol treatment resulted in approximately three-fold additional P4 production (270 ± 15 ng/dish/day or nM) compared to control, an effect which was abolished in the presence of

CH (86 ± 18 ng/dish or nM). In 3α-diol-treated cultures, P4 production was enhanced two-fold (162 ± 60 ng/dish/day or nM), an effect which was entirely prevented by CH.

5.5 DISCUSSION

The increased accumulation of P4 in culture medium by certain androgens (A-dione, 3α- and 3β-diol) and a norandrogen (19-NT) observed in our previous study, has been explored further in this study. Since the above stimulation could reflect either enhanced P4 biosynthesis and/or diminished metabolism of P4 to its principal inactive metabolite 20α-DHP (8), we studied P4 metabolism in the presence and absence of P4-stimulatory steroids. The negligible metabolism of radiolabelled P4 observed in our study of early placental explants is in accord with similar observations in term placenta (9). We did not observe any change in P4 metabolism after the addition of the P4-stimulatory steroids. This lack of alteration in P4 metabolism suggests that the stimulatory actions of androgens and norandrogen are related to increased biosynthesis rather than decreased catabolism of P4.

Although ACTH stimulation of adrenal P450scc activity is well established (10), regulation of this enzyme in placenta is still not clear. However, with the addition of a soluble substrate, 25-OHC (11), we observed an enhanced P4 production only when the concentration was 50 μM or greater. Similarly,

in the study of Winkel et al. (12), P4 secretion did not occur until the concentration of LDL cholesterol was 35 μg/mL (90 μM) suggesting that above this concentration there is a surplus of exogenous cholesterol which could serve as precursor for increased P4 biosynthesis. Consistent with our observation with 25-OHC (0.3%), a low conversion of P4 from labelled cholesterol (0.6%) was reported by Ryan (13). The low conversion found in our study indicates either limited entrance of exogenously added substrate or limited enzyme activity. Since the conversion of P4 from 25-OHC is augmented by treatment with 19-NT, A-dione and 3α-diol individually, it suggests that their P4 stimulatory effects are, at least in part, the result of an increase in placental P450scc activity.

An influence on the P450scc enzyme in the biosynthetic pathway of P4 is not unique to the above stimulatory steroids. Insulin in swine granulosa cells (14) and IGF-1 in human cytotrophoblast (15) are found to stimulate P4 biosynthesis by increasing P450scc activity. The P450scc is also found to be a common site of P4 regulation by estrogen in baboon placenta (16) as well as in porcine granulosa cells (17). Recently, Rabe et al. (18) reported that term placental P450scc is mainly inhibited by hydroxylated cholesterol derivatives formed as intermediates during cholesterol side chain cleavage. However, in contrast to our study, A-dione had little or no effect on the P450scc, a discrepancy which may be due to the placental age difference.

Using placental explants from early gestation, we found that radioactive P5 is converted into P4 in culture, thus indicating the presence of 3β-HSD in early placenta. This was also observed in the study of Rabe et al. (18). In the culture of human cytotrophoblast from late gestation, Nesler and Williams (15) have found a range of 1-10% of P4 conversion from exogenous pregnenolone, similar to our observation of 5%. This low conversion of radioactive P5 into P4 when radiolabelled P5 was incubated in the absence of any treatment was proabably due to dilution of radioactive substrate with the endogenous P5 pool.

Bioconversion studies of P5 to P4 demonstrated that all P4-stimulated steroids did not have the same effect. The stimulatory effect of 3β-diol on 3β-HSD was specific since the enzyme activity for P450scc was not increased following 3β-diol treatment. Since P450scc did not account for the entire stimulation of 19-NT, it could be that 19-NT has an action on 3β-HSD which is dose-dependent. Although we did not observe any effect of A-dione on 3β-HSD in early placenta, A-dione in late placenta was shown to inhibit 3β-HSD activity in a cell-free system (18) as well as in dispersed placental cells (20). Since cell breakage in dispersed cell preparations may affect enzyme stability, physiological interpretations may be misleading. In addition, difference in placental age may contribute to the discrepancy of the above observations as suggested for swine ovary (21), where the stimulatory action

of estradiol was found to be critically dependent on the maturational status of the parent Graafian follicles. Though 3β-diol increased the conversion of P5 to P4, 3α-diol had no effect.

In the presence of CH, basal P4 production was not blocked during the first 8 hr of incubation. This suggests either the absence of new protein synthesis or that the half-life of protein required for P4 synthesis is relatively long. Similarly, the stimulatory effect of ACTH on rat adrenal cholesterol uptake was not blocked by CH within a short period of 30 minutes (22). In 1986, Ogle (23) reported that nuclear processing of P4 receptor is not dependent on the synthesis of receptor protein, since CH treatment did not abolish the initial increase in binding activity induced by P4. Prolactin gene transcription was found to be stimulated within minutes of in vivo injection of estradiol (24,25). However, Shull et al. (26) observed prolactin gene transcription by estradiol within six hours of its addition to the cultured anterior pituitary cells; CH pretreatment had no influence on this induction, suggesting that estrogen regulates the expression of the prolactin gene at the transcriptional level through a direct action on the cells of pituitary gland and by a mechanism which is not dependent upon intermediary protein synthesis. Such a mechanism may account for 19-NT-stimulated P4 production over the first few hours in the presence of CH. It is also possible that the initial rapid P4 stimulation by

19-NT is mediated through a small pool of relatively stable protein which is resistant to the action of CH as suggested in the study of Dexter et al. (22). The possibility also remains that 19-NT causes initial P4 stimulation by stabilizing and/or retarding the degradation of the biosynthetic enzyme(s) as suggested by Gibori et al. (27) when estradiol treatment resulted in a dramatic increase in luteal P4 without concomitant alterations in enzyme content in their study.

During the longer period of incubation (24 hours), P4 stimulation by all the androgens (A-dione, 3α- and 3β-diol) as well as by 19-NT was affected by CH. This indicates a requirement for new protein synthesis. Since CH did not block the entire P4 stimulation by 19-NT, the CH-inhibitable process(es) is not the sole determinant for the stimulatory action of 19-NT on P4.

Although the underlying mechanism by which biosynthetic enzyme activities are augmented by the P4 stimulatory steroids, observed in this study, is not clear, a CH-inhibitable process may account for such an action. Our hypothesis is supported by the study of human term placenta by Nestler (28), who reported the requirement of new protein synthesis for the stimulatory actions of insulin and IGF-1 on 3β-HSD since their actions were found to be completely inhibited by concurrent treatment with CH. Our view is further supported by Picon et al. (29) who showed that the increase in 3β-HSD activity observed after dcAMP pretreatment was masked by CH in fetal

rat ovary. Alteration of P450scc enzyme activity is also reported to be dependent on a CH-inhibitable process. In a previous study (30) it was noted that cholesterol side chain cleavage in luteal mitochondria was controlled by a labile protein whose synthesis and activity were inhibited by CH. According to the study of Toaff et al. (17), P450scc is the locus of CH action in rat mitochondria where transport and binding of cholesterol to the side chain cleavage system are thought to depend on a protein factor. In the study of Holt et al. (31), P450scc activity was found to be increased by enhancing the translocation of cholesterol substrate to or across the mitochondrial membrane although the effect of CH on this process was not investigated. In baboon, Babischkin et al. (16) showed that estradiol maintained P450scc activity by maintaining cytochrome P450 content. It is possible that the P450scc activity in our study was stimulated by any of the above processes and mediated by a protein factor.

Expression of biosynthetic enzymes for P4 has been reported recently. In late human placenta, Ringler et al. (32) reported the involvement of cAMP in increasing the P450scc mRNA. In our previous study, with early human placenta, we did not observe any influence of cAMP on P4 production. Hormonal regulation of P450scc mRNA along with adrenodoxin (an iron-sulfur protein in mitochondria) mRNA in human placenta has been reported by Picado et al. (33). A recent study by Lorence et al. (34) described the expression of human

placental 3β-HSD in nonsteroidogenic cells.

Although we did not measure mRNA levels or protein content along with the biosynthetic enzyme activities in our study, in most of the previously reported studies, a close relationship among enzyme expression, content and activity was found. Couet et al. (35) recently showed the close correlation of 3β-HSD mRNA, protein content and activity levels in bovine ovary during the estrous cycle. In hypophysectomized rat ovary, prolactin caused a decrease in ovarian 3β-HSD mRNA content, accompanied by a similar decrease in 3β-HSD activity and protein levels (36). In the above study, slight stimulatory effects on 3β-HSD mRNA, protein content and activity level were also noticed in the presence of hCG. Similarly in porcine granulosa cell cultures, estrogen caused a parallel increase of P450 content along with P450scc activity (17). Induction of P450scc mRNA by estrogen and FSH has been reported (37).

Since in this study we have observed elevated enzyme activities of P450scc and 3β-HSD in the presence of P4-stimulating steroids, a parallel increase in the respective mRNAs and protein content seems likely. Such an increase in protein content may be due to a greater translation of preformed mRNA(s) for enzymes. However, the lack of correlation of 3β-HSD mRNA with increased 3β-HSD activities and protein content was also observed, suggesting the constitutive expression of 3β-HSD in rat ovary (36). Similar suggestions

were made for P450scc (37,38) as well as for aromatase cytochrome P450 (39) in rat ovary.

The recent cloning of human 3β-HSD cDNA (40) and the development of an antibody against purified human placental 3β-HSD (41) may provide the necessary tools for obtaining a better understanding of the regulation of P4.

In summary, this study in human placenta from early gestation showed that both P450scc and 3β-HSD are enhanced by 19-NT. While 3β-diol specifically acts on 3β-HSD, A-dione and 3α-diol act on P450scc. A role for a protein factor is suggested for P4 stimulation by all of the four steroids since their stimulatory effects were blocked by CH; however the early phase of 19-NT stimulated P4 was not dependent on new protein synthesis.

Table 5.1. Biosynthesis of [^{3}H]-P4 from [^{3}H]-P5 by early human placental explants

Treatment	n	% Conversion
none (control)	3	4.3 ± 2.0
19-NT (1 μM)	3	8.0 ± 0.7
,, (6 μM)	3	16.0 ± 4.0
,, (12 μM)	3	29.0 ± 1.0
3β-diol (1 μM)	2	4.5 ± 0.5
,, (6 μM)	2	12.0 ± 1.0
,, (12 μM)	2	20.0 ± 2.0
3α-diol (1 μM)	2	3.0 ± 1.0
,, (6 μM)	2	4.0 ± 1.3
A-dione (1 μM)	2	4.0 ± 0.8
,, (6 μM)	2	3.5 ± 1.5

n = number of placenta studied. Each placenta was cultured in duplicate.

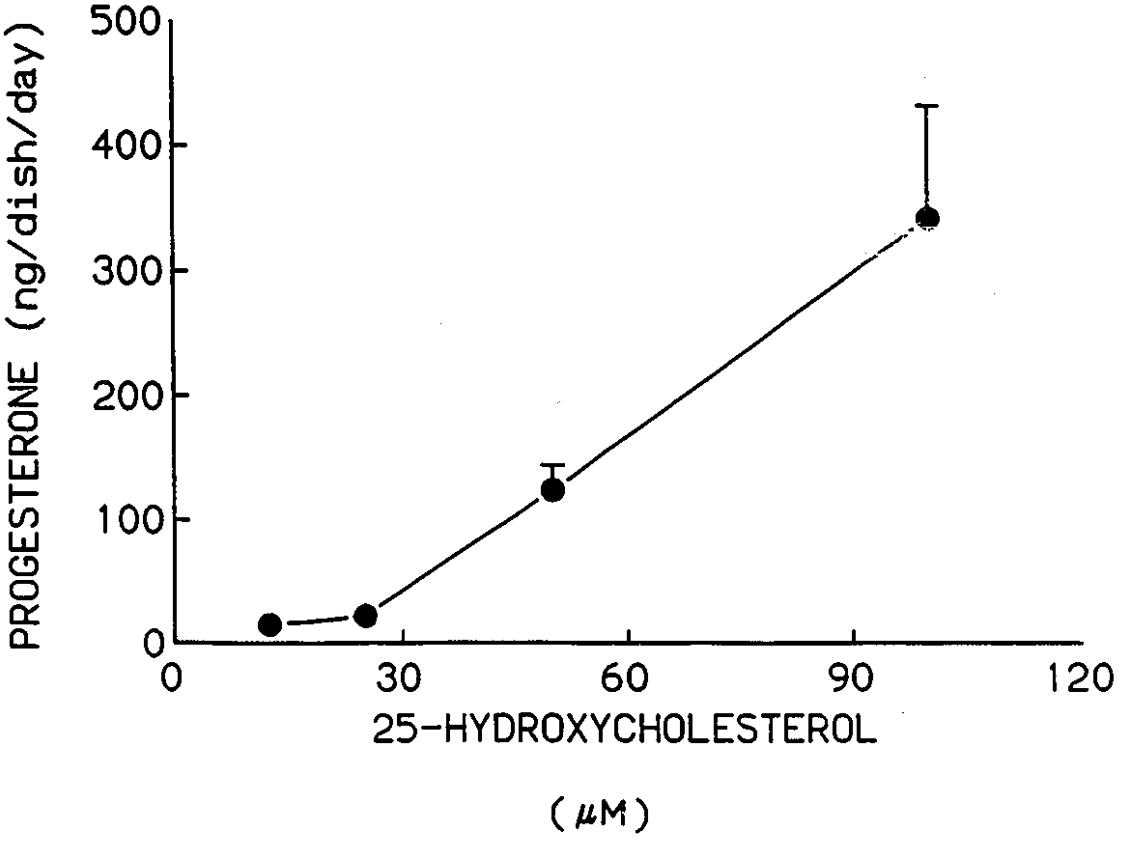

Fig. 5.1 Substrate-dependent conversion of 25-hydroxycholesterol to progesterone by placental explants incubated for 24 hours. Values represent the mean ± SE of quadruplicate observations from two different placental tissues.

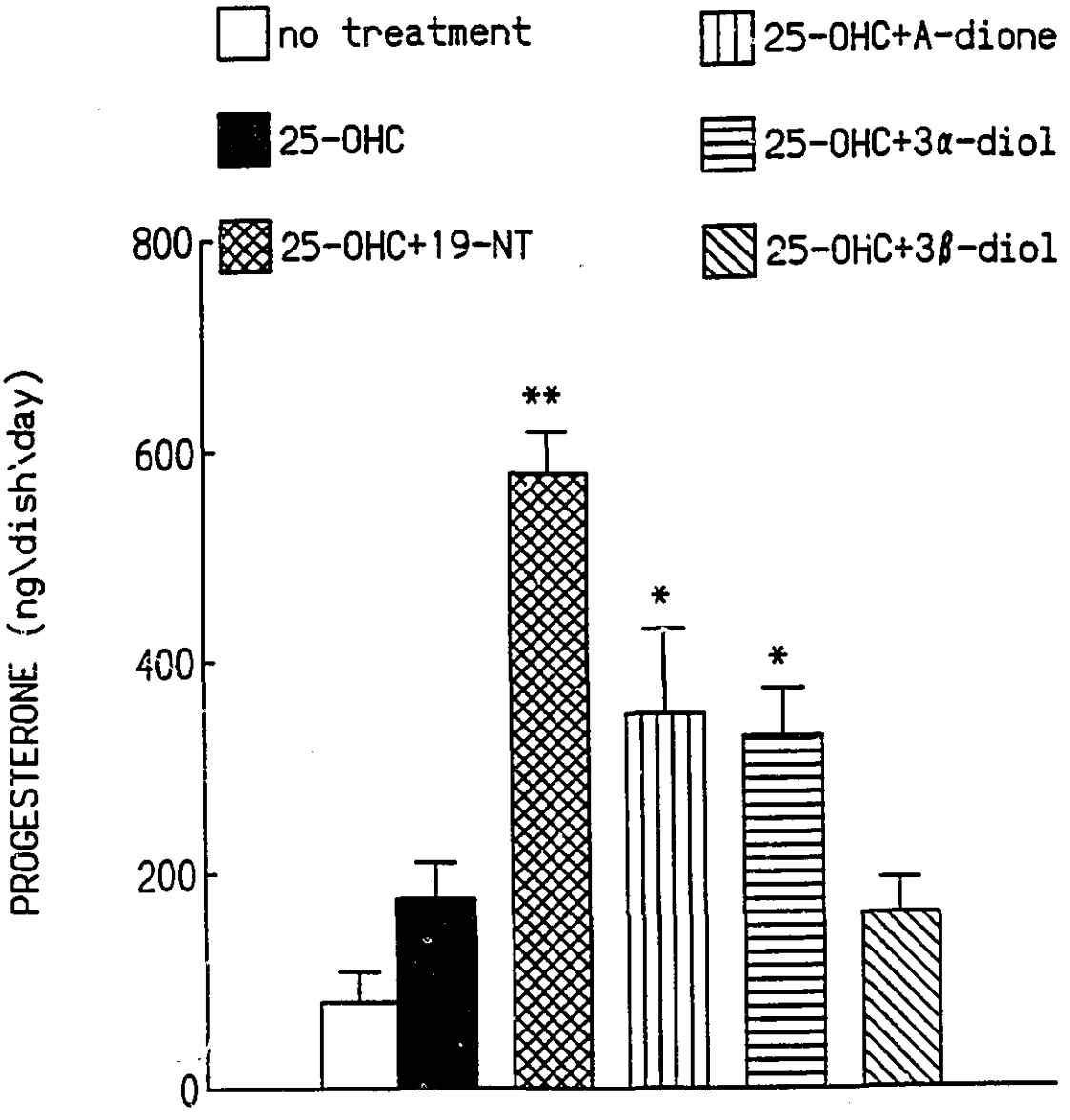

Fig. 5.2 Effect of 1 μm 19-NT, A-dione, 3α-diol and 3β-diol on the conversion of exogenously added 25-hydroxycholesterol (25-OHC) to progesterone in cultured media. Each bar indicates the mean ± SE of four observations from two different placental samples. none = culture without any treatment, 25-OHC = 25-hydroxycholesterol, 19-NT = 19-nortestosterone, A-dione = androstenedione, 3β-diol = 5α-adrostane-3β,17β diol; 3α-diol = 5α-androstane-3α,17β diol. * = $P < 0.05$, ** = $P < 0.01$ compared to the level obtained only with 25-OHC without any treatment.

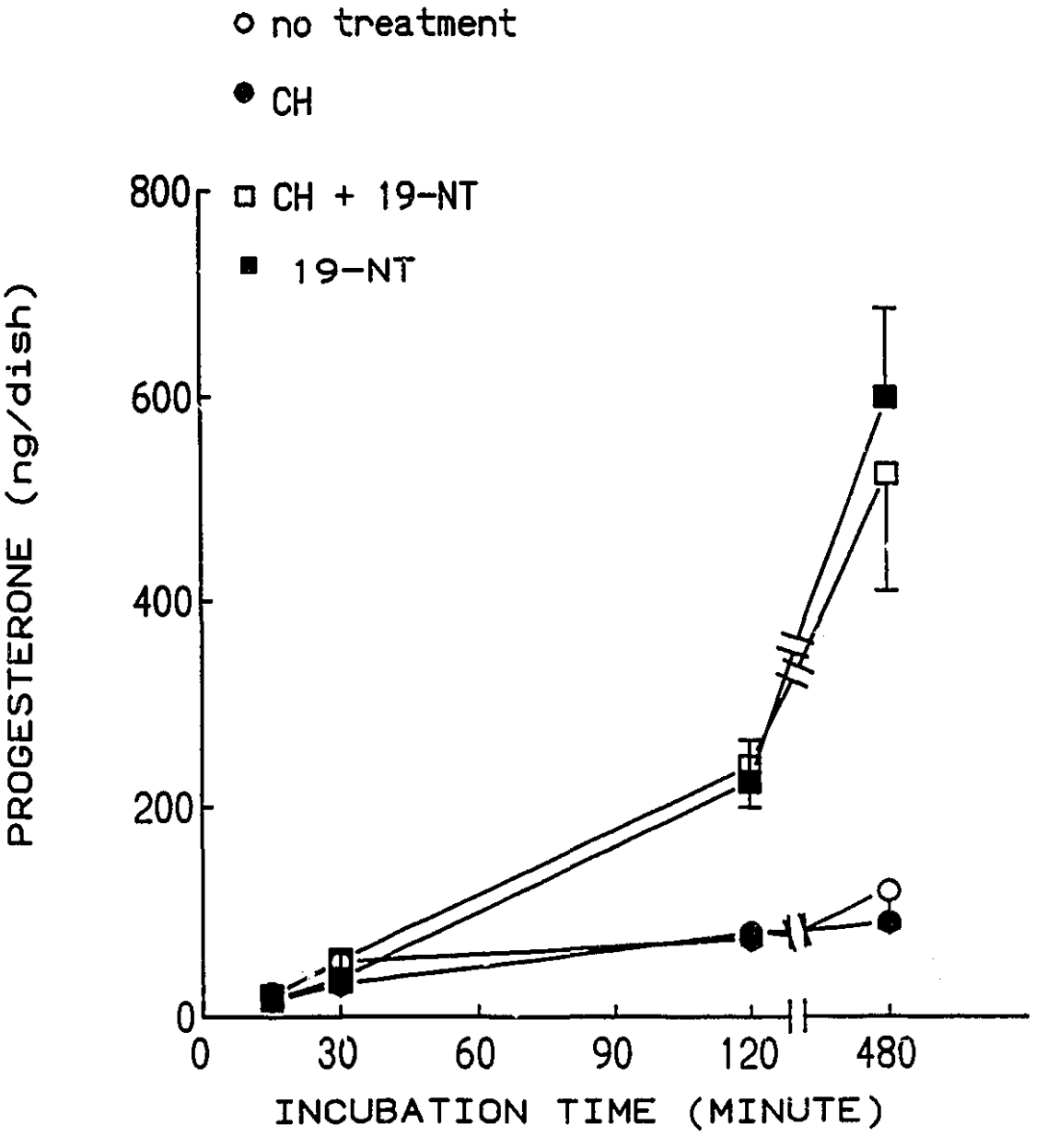

Fig. 5.3 Effect of cycloheximide (1 mM) on 19-NT (1 μM) treated cultures at different times of incubation. Each point is the mean ± SE of three observations from three different placental samples. CH = cycloheximide, 19-NT = 19-nortestosterone.

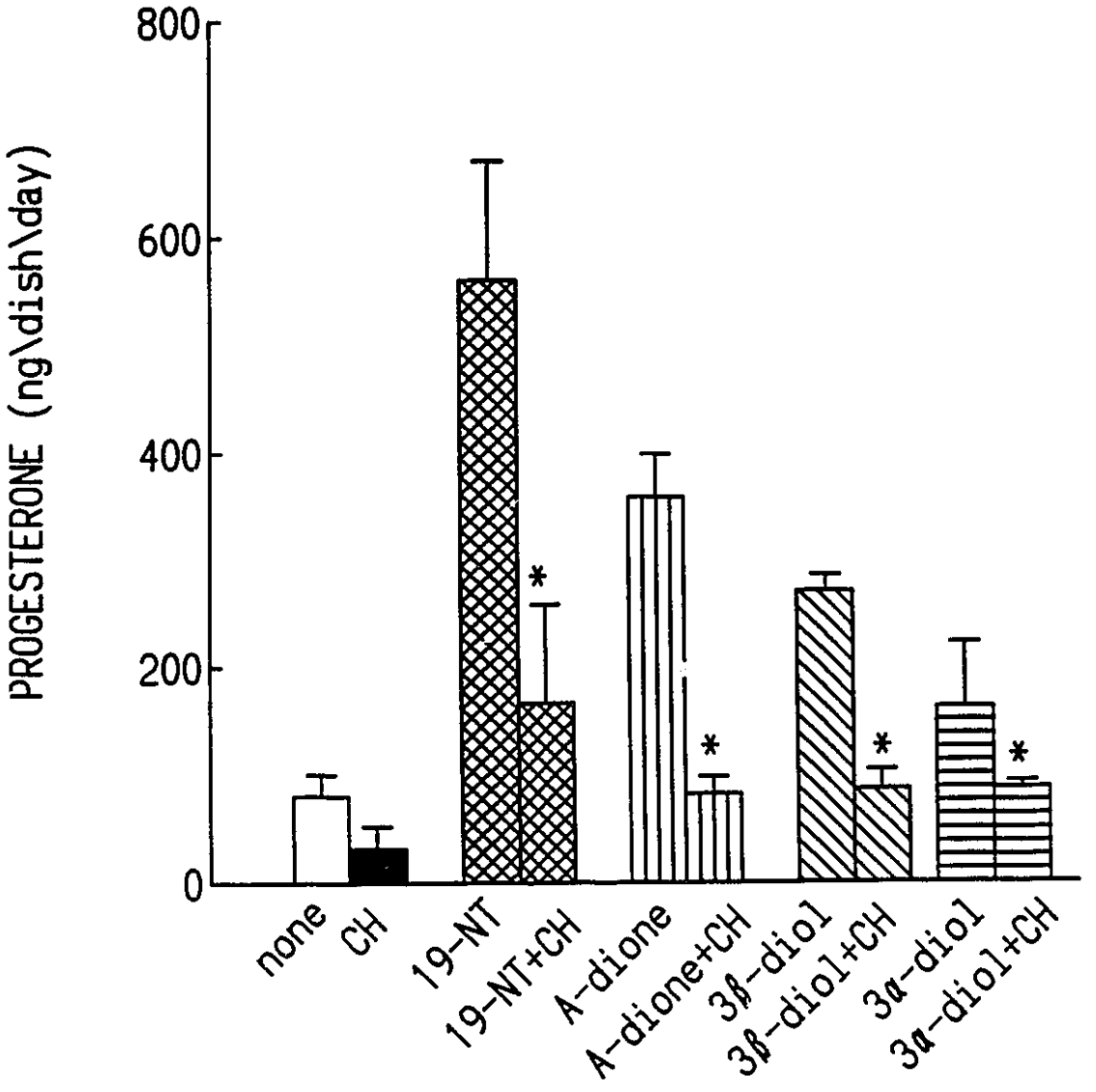

Fig. 5.4 Effect of 19-NT, A-dione, 3α- and 3β-diol at 1 μM concentration, on the progesterone production in culture, incubated for 24 hours in the presence or the absence of cycloheximide (1 mM). Each bar is the mean ± SE of the triplicate observations from three different placental samples. none= no treatment, CH = cycloheximide, 19-NT = 19-nortestosterone, A-dione = androstenedione, 3β-diol = 5α-androstane-3β,17β diol; 3α-diol = 5α-androstane-3α,17β diol. * = P < 0.05 compared to respective treatment in absence of cyloheximide.

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CHAPTER VI

EFFECT OF MATERNAL SERUM ON VIABILITY AND FUNCTION OF EARLY HUMAN PLACENTAL EXPLANTS

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6.1 ABSTRACT

Fetal bovine serum (FBS) is frequently used to supplement chemically defined media when studying placental explant cultures. However in vitro production of hormones is usually declining by the second or third day and is short-lived (7-10 days). In this study we explored the use of human maternal serum (HMS) from early gestation as the medium supplement for explants obtained from the same patient. Early placental hormone production was compared using two concentrations of FBS and HMS added to the chemically defined nutrient mixture Ham's F-10. On the third day of incubation, β-hCG production (mean ± SE) in the presence of 10% FBS was 8.2 ± 2.9 IU/mL and with 10% HMS it was 29.2 ± 3.7 IU/mL ($P < 0.005$), a more than 3-fold increase. Estradiol (E_2) production in the presence of 10% FBS was 0.31 ± 0.06 ng/mL and in the presence of 10% HMS was 3.4 ± 0.76 ng/mL ($P < 0.001$), a 10-fold increase. Progesterone (P4) production in the presence of 10% FBS was 3.0 ± 0.4 ng/mL and in the presence of 10% HMS, it was 36.0 ± 7.3 ng/mL ($P < 0.001$), more than ten-fold; thus by day 3 the difference was more than 10-fold. When the serum concentrations were increased to 40%, the results in all cases were similar to those at 10%. Thus for all 3 hormones, production was significantly higher (3- to 10- fold) when 10% HMS was used and was not further increased by increasing the serum concentration. Preliminary characterization studies revealed

that the stimulatory activity of HMS is heat-labile, neither extractable into organic solvent (diethyl ether) nor dialyzable, suggesting that stimulatory activity of HMS is protein in nature. In a long term incubation, compared with FBS (7 days), HMS permitted longer survival of culture up to 20-30 days, judged histologically and biochemically. There is a strong correlation between metabolic and histologic viability of placental culture for both of FBS ($r = 0.90$, $P < 0.02$) and HMS ($r = 0.74$, $P < 0.05$). We conclude that HMS is a better supplement for early human placental cultures than is FBS and that protein component(s) of HMS may be important for human placental development and function in vivo.

6.2 INTRODUCTION

Human placental explant cultures provide an important material for the investigation of the hormonal regulation of this tissue. Although recent advances have been directed toward the development of chemically defined media selective for specific tissues, serum is used widely as an essential component of both cell and organ culture. Numerous attempts have been made to define an optimal physiological model for in vitro production of hormones with different degrees of success. Tissue media have been supplemented with various concentrations of serum (5-20%) usually from animal sources, to increase basal hormone production. Several investigators

have used commercially prepared nutrient mixtures, such as Ham's F-10, supplemented with 10 to 20% FBS as their *in vitro* culture medium. However, functional instability of explant cultures with short term survival has limited the applicability of such media (1-3). This suggests that the nutritional factors of Ham's F-10 + FBS are not optimal. Although *in vitro*, it is not possible to reproduce *in vivo* conditions exactly, it is desirable to achieve as physiological a state as possible. In an attempt to achieve this, the use of first trimester human maternal serum as the supplement for the culture of human trophoblast explants was explored.

6.3 MATERIALS AND METHODS

After obtaining informed consent, early (8-10 weeks gestation), and midterm (12 - 20 weeks gestation) human placental tissues, dated from the last menstrual period and/or foot-length [4]) human placental tissues were obtained from the pregnancy termination units of the Montreal General Hospital and other local hospitals at the time of dilatation and evacuation for socio-medical reasons. All were from normal healthy women. Term placental samples were obtained after normal delivery.

Blood samples were collected from the same group of patients in early pregnancy. After centrifugation at 4°C, the sera were pooled and refrigerated until used. Two late sera

were obtained from normal pregnancy at term and two non-pregnant sera were obtained from otherwise normal women attending a fertility clinic, before any hormone treatment.

Powdered Ham's F-10 and fetal bovine serum (FBS) were purchased from Grand Island Biological Co., Burlington, Ontario. Amphotericin B was obtained from Squibb (Montreal), penicillin G from Glaxo Laboratories, Montreal, and gentamycin sulphate from Schering, Pointe Claire, Quebec. Small plastic culture dishes (60x15x2 mm) were obtained from Falcon plastics, Los Angeles, CA. Supporting grids (2.5x2 cm) were from Johnson wire works, Montreal, Quebec. Diethyl ether used for extraction of steroids was purchased from American Chemical Company, Montreal.

Seamless dialysis tubing (3.1 cm wide) with a molecular weight cut-off point of 12,000 daltons was obtained from Fisher Scientific Co., Montreal.

Unlabelled steroids were from Sigma Chemical Company, St. Louis, MO. All steroids were dissolved in ethanol and stored at -10°C. Radioactive steroids [2, 4, 6, 7-^{3}H(N)]estradiol, SA 115 Ci/mmol, and progesterone [1, 2, 6, 7-^{3}H(N)], SA 112 Ci/mmol were obtained from New England Nuclear Corporation, Boston, MA. These were diluted with ethanol to a concentration of 50-100 uCi/mL and stored at -10°C.

Tissue preparation

Placental tissue kept on ice was processed within one

hour of removal. Tissues were rinsed thoroughly in cold saline to remove blood, and villous tissue was minced with sharp scissors and scalpel. Ten small pieces of tissue, each about 1-2 mm^3 (approx. 1 mg each) were placed on two metal grids in a culture dish containing 3 ml culture medium composed of Ham's F-10 + antibiotics (penicillin G: 200 IU/mL; amphotericin B: 5 μg/mL ; gentamycine sulfate: 40 μg/mL) ± serum (FBS or HMS). For each culture, control and experimental dishes were prepared from a single placenta or pool of 2-3 placentas of the same gestational age, and incubated with a single batch of FBS or HMS.

Preliminary characterization of HMS

Commonly used biochemical tests were carried out by the routine hospital laboratory on various pools of HMS and FBS for comparison. To investigate the nature of the influence of HMS on culture, aliquots from a pool of HMS from early gestation were treated in one of the following ways: (a) heated for one hour at 60°C, (b) extracted once with five volume of diethyl ether and (c) dialyzed against 0.9% saline at 37°c for 24 hours using dialysis tubing with a 12,000 molecular weight cut-off. Duplicate dishes were treated separately with heated HMS, solvent extract, aqueous residue, dialysate and dialysand of HMS, for 24 hours on day two. Nonpregnant and late pregnancy sera were used similarly. All were added at a concentration of 10%.

Incubation

Placental cultures were incubated in a humidified environment of 5% CO_2 in air at 37°C. In a few instances the incubation period (usually 1-7 days) was prolonged to 45 days. Culture media were changed daily for short cultures and every 1-2 days for long cultures. The collected media were stored at -20°C for subsequent hormone analysis. For each experiment, duplicate dishes were cultured from single placenta.

From the long cultures, two explants from each dish were collected at intervals and immediately immersed in 10% formalin for histological study. Fresh placental tissue fixed similarly served as control.

Extraction

For estrogen and progesterone analyses, sample media were extracted once with five volumes of diethyl ether. Extracts were dried and redissolved in ethanol, and stored at -20°C until assayed. Recoveries of tracer hormones added before extraction were 90-95%; no corrections were made for recovery.

Hormone analysis

Progesterone was determined by competitive protein binding to the progesterone-binding globulin of pregnant guinea pig serum (5,6).

Estradiol was determined by conventional radioimmunoassay using dextran-coated charcoal to adsorb the unbound fraction; the antiserum used was kindly provided by Dr. Hamish

Robertson, Ottawa, and was specific for estradiol, the highest cross-reactivity among related steroids being for estrone (5% at 50% displacement).

The hCG assay was done using a commercial kit specific for the beta subunit (Immunocorp,, Montreal, Quebec).

All samples from a given experiment were assayed simultaneously to avoid interassay variations which were less than 12% for the assays of all 3 hormones. Intraassay reproducibility ranged from 5-7%. Media without tissue were used as controls along with each type of culture and values for the media without tissue were subtracted from the respective results of experiments with tissue.

Tissue histology

Following routine staining of the tissue with haematoxylin and eosin, assessment of viability for each explant was made by a pathologist unaware of the treatment, who graded it from 0-4 as follows:

0: necrotic, complete loss of integrity of the villi, lack of differential staining of nuclei, cytoplasm and core of the villi with the presence of strong acidophilic staining all over the tissue.

4: completely healthy tissue, indistinguishable from that freshly obtained, with integrity of the villi showing clearly the outer syncitium, underlying cytotrophoblast and internal core tissue with the presence of differential staining in the

nuclei and cytoplasm as well as in the interior of placental villi.

Scores of 1 and 2 were intermediate, according to the reduction or variation in intensity of staining of trophoblast.

Statistical analysis

The data were analyzed for differences and variances by Student's t-test. A difference was considered significant if $P < 0.05$. Correlations were calculated using Pearson's correlation coefficient.

6.4 RESULTS

Progesterone production by early explants in Ham's F10 in the absence of serum is compared with that in the presence of FBS 10 % in Fig. 6.1. There was a gradual 10-fold decrease from day one to day four, with levels in the absence of serum about one-half those in the presence of FBS. A similar decrease was observed in mid-term and late gestational explants maintained with FBS (6.2). Estradiol showed similar pattern, with a 5-fold fall over the first 3-4 days in culture, in early and late gestation explants in the presence of 10% FBS (Fig. 6.3).

The effects of HMS and FBS (10% and 40%) on progesterone secretion by early placental cultures are compared in Fig. 6.4. At both concentrations, the progesterone production was

significantly higher ($P \leq 0.05 - 0.01$) with HMS (108 ± 9.2 ng/dish vs 70 ± 5.3 on day one at a concentration of 10%; 70 ± 3.3 vs 24 ± 4.5 on day 2; 145 ± 30 vs 15 ± 1.2 on day 3). When the concentrations of both sera were increased from 10% to 40%, the results were similar.

Figure 6.5 compares estradiol secretion in the presence of 10% HMS and 10% FBS. With HMS the production after one day was 7-fold higher (9.5 ±0.5 vs 1.4 ± 0.05, $P \leq 0.05$) than with FBS and it increased by a further 30% over the next 2 days(10 ± 2 vs 0.8 ± 0.04, $P \leq 0.05$ on day 2 and 13 ± 1 vs 0.7 ± 0.1, $P \leq 0.01$ on day 3). On the contrary, when explants were cultivated in the presence of 10% FBS for the same time span, a gradual decline of E_2 production (1.4 ± 0.05 to 0.7 ± 0.1 ng/dish) occurred. Again the results using 40% serum were similar to those at 10% and no further increase in estradiol production was observed.

In addition to the steroid hormones (E_2 and P4), the effects of HMS and FBS were also studied for a protein hormone, hCG (Fig. 6.6). Although not significantly different on day 1, the production of hCG on day 2 was much greater ($P \leq 0.01$) in the presence of HMS 10% or 40% and this difference persisted on day 3.

Since production of three major hormones by early placenta was increased in the presence of HMS over the short culture period of 3-4 days, it was of interest to know how long production, and presumably viability, could be prolonged.

The results of the long-term cultures for early gestation explants are shown in Table 6.1 and Fig. (6.12-6.15). No explant preparation from early placental tissue showed decidual contamination on histological study. Viability of cultured explants was assessed by comparing the microscopic appearance of the explants with that of fresh placental tissue at the same gestational age.

Histologically, control cultures in the absence of FBS remained viable for 5 days after initiation of culture, with a gradual decrease of viability from day one to day 7. A similar pattern of decreasing viability from day one to day 7 was observed in cultures in the presence of FBS. By contrast, in the presence of 10% HMS, cultures showed no tendency to degenerate on day 7 and viability persisted for as long as 30 days, a 6-fold prolongation over that obtained with FBS (Table 6.1).

Metabolic viability of the same cultures was assessed by hormone productione in the media (Fig. 6.7a,b,c). Progesterone production (ng/dish/day) (Fig. 6.7a) was found to decrease gradually from day one to day seven in the absence of serum or in the presence of FBS and was negligible by day ten, suggesting a high correlation between metabolic and histological viability ($r= 0.9$ $p < 0.025$). Similarly progesterone output (ng/dish/day) in the presence of HMS 10% remained significant until day 35 although levels were declining; however they did not approach base-line levels until after 30 days, again

showing a positive correlation ($r = 0.74$, $p<0.05$) with the histology.

Similarly, estradiol (Fig. 6.7b) and hCG production (Fig. 6.7c) were significant ($P \leq 0.05$) for more than 30 days in the presence of HMS.

To explore the nature of its stimulatory activity, early gestational HMS was treated in various ways (Fig. 6.8). When the explants were cultured with heated 10% HMS, the stimulatory activity as indicated by progesterone production (ng/dish-/day) by early placental cultures was no longer present. Further investigation showed that the stimulatory activity of HMS was associated with the residual aqueous phase but not with the fraction extractable with diethyl ether. Also, the stimulatory activity was associated with the non-dialyzable component, suggesting its association with substance(s) of molecular weight in excess of 12,000 daltons.

By contrast with serum obtained during pregnancy, the effect of non-pregnant serum (10%) was similar to that of 10% FBS (Fig. 6.9). However, the stimulatory activity of late maternal serum was not significantly different from that of early HMS. The early HMS was equally stimulatory to preterm and term placental progesterone production (Fig. 6.10).

Progesterone production after adding 1 μM 19-nortestosterone to 10% FBS was almost 2-fold higher than that of HMS 10% (Fig. 6.11). When the 19-nortestosterone was added to HMS 10%, the effects were additive.

Relevant constituents of early gestation HMS and of FBS are compared in Table 6.2. The amount of total protein and albumin in HMS were double those in FBS. The content of LDL cholesterol (the principal progesterone precursor), was about twenty fold higher in HMS than FBS. The concentration of the estrogen precursor DHAS was undetectable in FBS whereas it was 12 μMol in HMS. The level of insulin in HMS was about ten-fold higher than that in FBS. The HMS also contained 4-8 times more progesterone and estradiol than FBS. As expected, hCG was very high (mean ± SE: 70 ± 2 IU/mL) in HMS but undetectable in FBS, since this protein is not produced in the cow.

6.5 DISCUSSION

As a model of physiological function *in vivo*, the validity of *in vitro* tissue culture has always been disputed due to the alteration of the environment. The provision of an appropriate culture medium is fundamental for the expression of differentiated function in any biological system. Hsu (7) described how a minor alteration of the feeding regimen could cause a change in the karyotype of mouse fibroblasts, suggesting an extraordinary sensitivity of aneuploid cells to variations in culture technique . It seems reasonable to attempt to simulate in so far as possible the environment from

which the tissue was removed. During pregnancy, human placenta is exposed to both the maternal and fetal circulations, however trophoblast is unique in having direct contact with maternal blood in the intervillous space, without having a barrier composed of a layer of endothelium and basement membrane, as on the fetal side. While it is impossible to obtain enough human fetal blood for culture purposes, it is relatively easy to obtain human maternal serum.

In this study we have shown that incorporation of HMS into the culture medium increases the expression of the differentiated function of human trophoblast in culture when compared to that of fetal bovine serum. We are unaware of any previous similar report.

Placental cultures from early, mid and late gestations all showed decreasing progesterone, estradiol and hCG production in the presence of FBS in the first week of culture, which was prevented by HMS. There appeared to be no marked influence of placental age on survival of explants in culture, and no obvious difference between early and late gestational HMS.

Our observation that progesterone production did not change with placental age is consistent with the findings of others (8), indicating that increase in maternal serum progesterone concentration during late gestation is due to increase of functional mass of placental tissue rather than to an increase in capacity per unit mass. Similarly, the

production of estradiol in late gestation did not appear to alter appreciably with age.

While it is desirable to use chemically defined media for tissue culture, it is rarely possible to dispense entirely with serum supplements. Fell (9) reported that the use of chemically defined medium without serum supplementation was not practically satisfactory since few tissues retain their normal architecture in serum-free medium. Veldhuis et al. (10) found that in the absence of serum there was inhibition or minimal alteration of progesterone accumulation with addition of estradiol in swine granulosa cell cultures. On the contrary, with serum supplementation (1-10%), progesterone production stimulated by estradiol was significantly enhanced above basal, suggesting that the ability of estradiol to stimulate progesterone biosynthesis was critically dependent upon the presence of serum in swine granulosa cell cultures. Recently Branchaud et al. (11) have suggested that human placental monolayer cultures can be satisfactorily maintained in a serum-free medium. In their study the output of human placental lactogen, hCG and aromatase activity were found to be 40%, 50% and 20%, respectively, less in serum-free medium compared to medium enriched with FBS. Thus although the differences were small, it appears that serum, and particularly human serum, may contain still unrecognized substances which enhance function and viability in culture. Since the protein of serum acts as a stabilizer for many substances

(12), the lack of protein may destabilize some substances in chemically defined media, making them less effective.

In our study, production of all three hormones (progesterone, estradiol and hCG) was increased in the presence of HMS compared to FBS, suggesting that human trophoblast has its own characteristic nutritive requirements provided by HMS.

Our findings for the patterns of hormone secretion for explants cultured in Ham's F-10 + FBS are similar to those of others (13-16). Thus Branchaud et al. (13) also found low baseline secretion of estradiol, while the decline we noted for progesterone over the first few days of culture was similar to that found by Paul et al. (14). That for hCG is similar to that reported by Ahmed et al. (15) and Winikoff et al. (16), i.e. a fall on day 2, with recovery by day 3.

HMS was also able to prolong the useful life of placental explants by about 6-fold, as judged both histologically and functionally. Biggers et al. (17) showed that necrotic changes may occur in the centres of explants of embryonic chick heart within 48 hours; we also found that such changes occurred in our placental explants cultured in FBS; however such changes did not occur in the placental explants until about 20 days or more when cultured in HMS. Thus the use of HMS permits a more physiological environment to be achieved for a prolonged period of time.

In our study, when explants in FBS showed rapid degeneration at the cellular level compared to that in HMS, our

observations did not exclude the possibility of similar differential genotypic changes occurring at the nuclear level. However, since in our study human placental capacity to adjust to the culture environment was facilitated in the presence of HMS, placental culture with HMS may have wide appalication in the study of hormonal regulation.

In accord with our study, early human placental explants in culture with FBS 10% showed gradual necrosis after 3 days of culture and could not be maintained more than a week (18). In the above study, it was also shown that the viability of placental explants treated with various steroids (progesterone, DHA, and cortisol), singly or in combination, could be prolonged as long as 20 - 30 days.

Ahmed et al. (18) showed that hCG production could be increased by adding various steroids (progesterone, DHA, cortisol); while these are present in HMS, and would be extracted into diethyl ether, the important stimulatory activity of HMS appeared to be associated with the protein fraction rather than with the solvent-extractable fraction.

The importance of protein in the maintenance of <u>in vitro</u> culture has been considered for many years. Biggers et al. (17) suggested that protein in the medium has a protective effect on cells in culture, perhaps by adsorption on to the cell surface. This group also found that the addition of non-specific macromolecular substances prevented the rapid loss of motility and death of spermatozoa; and albumin appeared to

preserve the viability of segmenting mouse ova for 24-48 hours.

Recent studies have emphasized the need for various growth factors (19). However the factors controlling the growth and maintenance of placenta are poorly understood. Wu et al. (20) described growth factors of 27,000 and 41,000 daltons in the conditioned medium from human term placental tissue. Recently Riopel et al. (21) reported a heat-labile macromolecule (> 30,000 daltons) associated with growth-promoting activity of human placenta. Similarly a placental growth factor of 34,000 Da has been described by Sen Mujumdar et al. (22). These observations suggest that pregnancy-related protein(s) (23) could play an important role in placental development. This suggestion is supported by our observation of the importance of circulatory protein from maternal serum in the maintenance of viability and the expression of differentiated endocrine functions.

Since in our study non-pregnant serum lacked the stimulatory action of HMS from early as well as late gestation, as indicated by progesterone production in culture, it seems that some pregnancy-specific but as yet undefined protein(s) present in HMS may be important for placental development <u>in vivo</u>. Their mode of action is entirely unknown and can only be speculated upon at this point. Many receptors might be involved, such as those for epidermal growth factor (24) and insulin growth factor (25), which are known to be present in

human placenta.

Analogous to our study, the plating efficiency of Hela-S3, Hela-Gey, Chang-liver and Maben strains of human epithelial cells was found by Murphy et al. (26) to drop sharply when plated in heterologous sera containing calf or horse serum compared to that in homologous serum containing only human serum. In their study it was also reported that cell cultures propagated in calf serum were more sensitive to respiratory virus infection than those grown in human serum. Andrews and Phillips (27) observed that HeLa cells respired and formed lactate at a greater rate when cultured in medium containing human serum in comparison to the same cells grown in horse serum. It was postulated previously (28) that homologous protein facilitates the synthesis of new building units in the living cell since the configuration of the polypeptide chain in homologous protein is more closely related to that of the tissue protein. This concept may be relevant for our observation of human maternal serum protein supporting human placental survival for the expression of its endocrine functions.

Although we did not measure growth of placental explants cultured in HMS, no noticeable increase in size or any outgrowth from the edges of explants was observed. Therefore, it appears that the protein of HMS is more closely associated with maintenance of tissue integrity than directly with growth.

Table 6.2 shows that, in comparison to FBS, HMS is enriched with regard to protein as well as hormones. At present, the nature of the protein responsible for the stimulatory action of HMS is not known. Although HMS contains considerable amounts of low density lipoprotein cholesterol and dehydroepiandrosterone sulfate, the known precursors for progesterone (29) and estrogen (30), respectively, the stimulatory action of HMS in our study did not appear to be accounted for by these steroid precursors. Since expression of all three products of syncytiotrophoblast (progesterone, estradiol, and hCG) was enhanced in the presence of HMS, possibly a common factor(s) may be involved in the stimulation of all three hormones rather than involvement of individual precursors.

For all three hormones, the effects of both HMS and FBS at high concentration (40%) were not improved over those at the lower concentration (10%). This may be due to the limitation of the *in vitro* culture technique in the absence of the circulation. The metabolites in culture are not flushed away as quickly as *in vivo*, where flow is continuous.

The effect of HMS as indicated by placental progesterone production was not influenced by the gestational age of maternal serum or placental samples, suggesting that the factor(s) associated with the stimulatory effect of HMS is available for placental development throughout pregnancy.

The stimulatory effect of 19-nortestosterone on proges-

terone production as found previously when culture was supplemented with FBS 10% (31) was also observed in the presence of HMS 10%. Since the combined effects of HMS and 19-nortestosterone on progesterone production were additive, their effects are probably not mediated through a common mechanism.

Although it is difficult to extrapolate in vitro results to the in vivo situation, the present study suggests that HMS to which placenta is exposed in vivo provides appropriate support to placental development for the expression of differential function during pregnancy.

Since both steroid and peptide hormone production was best achieved by supplementing chemically defined medium Ham's F-10 with HMS 10%, we conclude that, for human trophoblast in culture, HMS is a better support than FBS. Moreover, for many of those investigators who work with human placenta, HMS is much more economical than FBS. Since it is possible to establish long-term cultures in the presence of HMS, its use should provide a better in vitro model for various kinds of investigations involving chronic as well as acute responses of placental tissue in culture.

TABLE 6.1

Tissue histology score

Control (Fresh tissue before culture) = 4			
Day	F-10 + FBS 10%	F-10 + HMS 10%	F-10
1	3	4	4
3	2	3	3
5	1	3	1
7	0	3	-
15	0	3	-
30	0	2	-
45	0	0	-

\- = not done
4 = completely healthy tissue
0 = completely necrotic tissue

TABLE 6.2

Comparison of the constituents of HMS and FBS

Constituents	HMS (mean ± SE, n)	FBS (mean ± SE, n)
Total protein (gm/L)	100, 73	40, 41
Albumin (gm/L)	50, 45	23, 26
Total cholesterol (mM)	4.1 ± 0.2 (4)	0.8 ± 0.1 (4)
LDL (mM)	3.4, 2.4	0.16, 0.24
HDL (mM)	1.0, 1.6	0.2, 0.3
DHAS (μM)	12, 7.2	ND, ND
Cortisol (nM)	649	6
Insulin (pM)	102, 120	27, 20
ß-hCG (IU/mL)	72, 68	ND, ND
Progesterone (ng/mL)	28, 32	6.8, 7.2
Estradiol (ng/mL)	5.0 ± 0.1 (6)	0.6 ± 0.1 (4)

Each observation is the mean of the number of replicate determinations (each done in duplicate) indicated in the brackets. Where there were only two determinations (each done in duplicate) both values are given. ND = not detectable

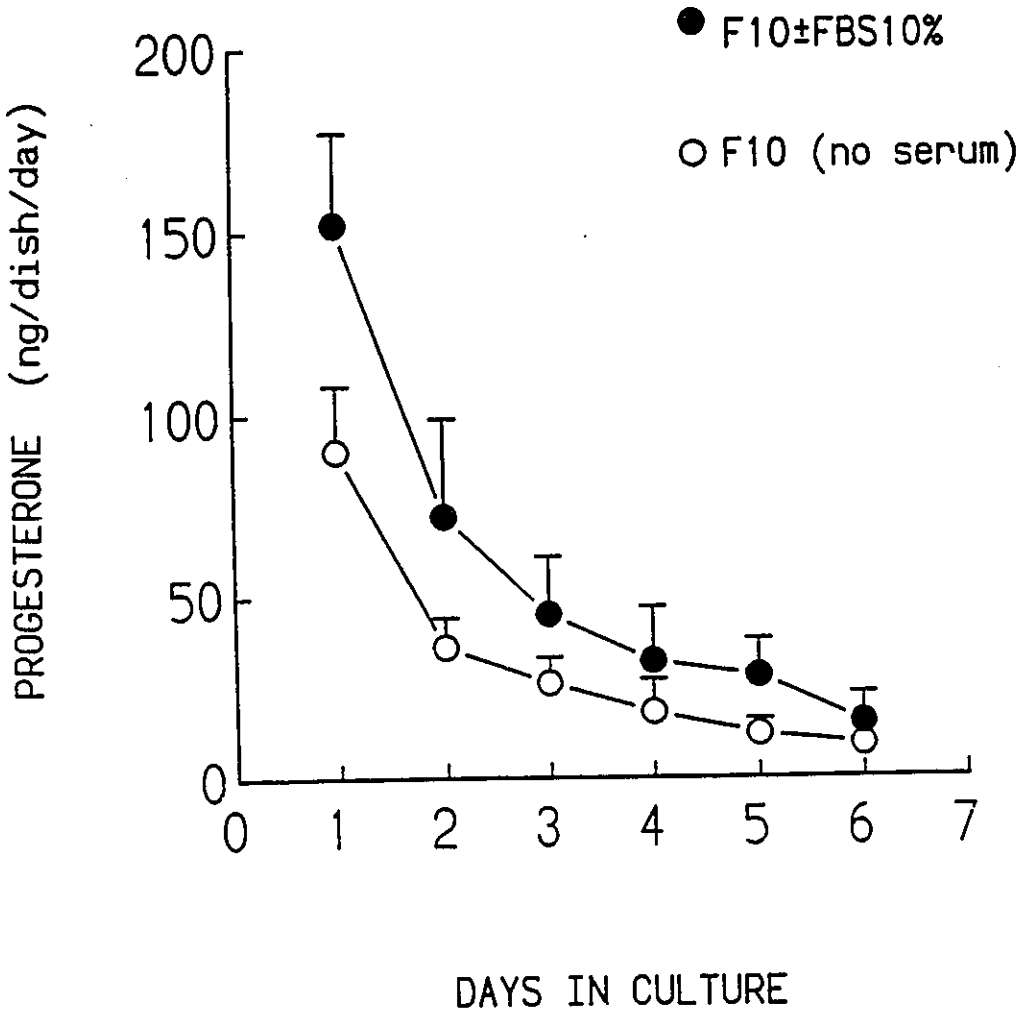


Fig. 6.1 Basal progesterone production in early placental culture with Ham's F-10 in presence or absence of FBS 10%. Each data point indicates the mean ± SE of quadruplicate determinations. (duplicate dishes from two different placentas).

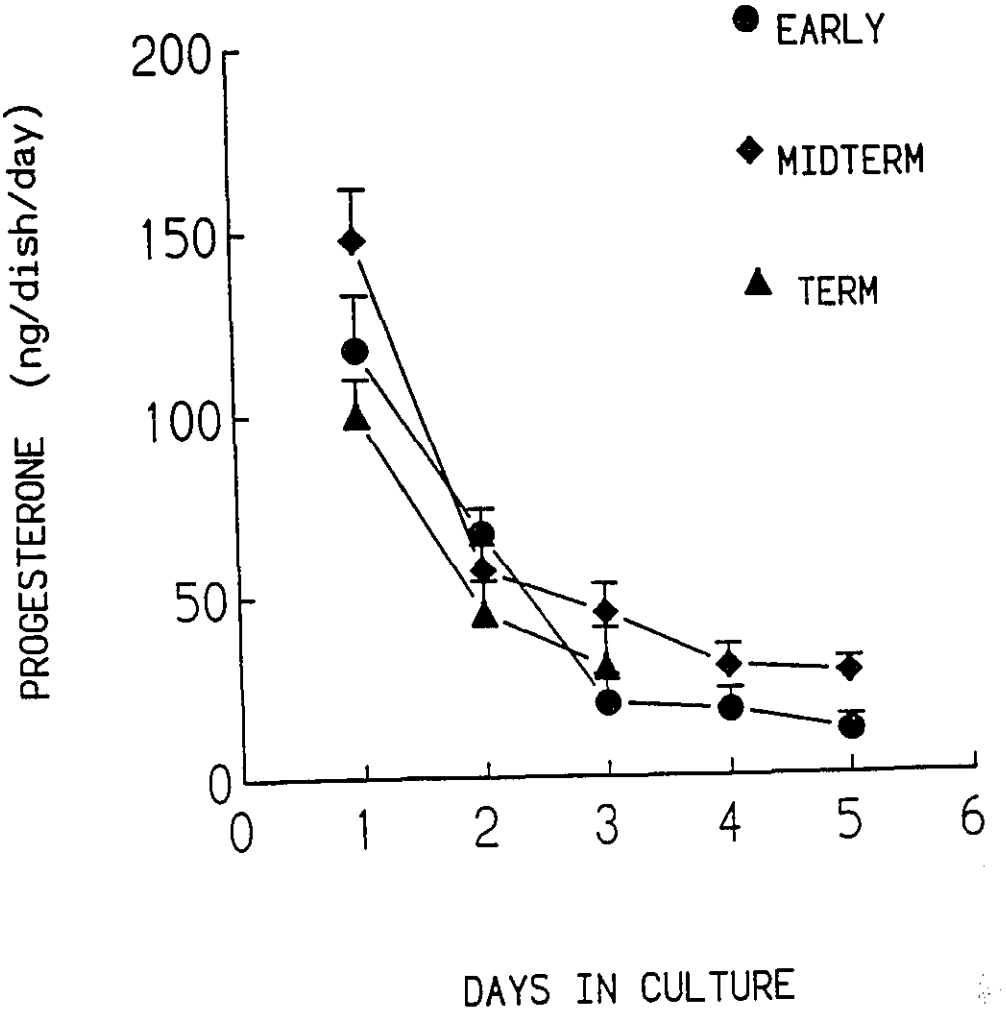


Fig. 6.2 Basal progesterone production from early, midterm and term placental culture with Ham's F-10+FBS 10%. Each data point indicates the mean ± SE of quadruplicate observations (duplicate determinations from two separate dishes) from early, mid- and term placental tissue. Two different placentas were used for each point.

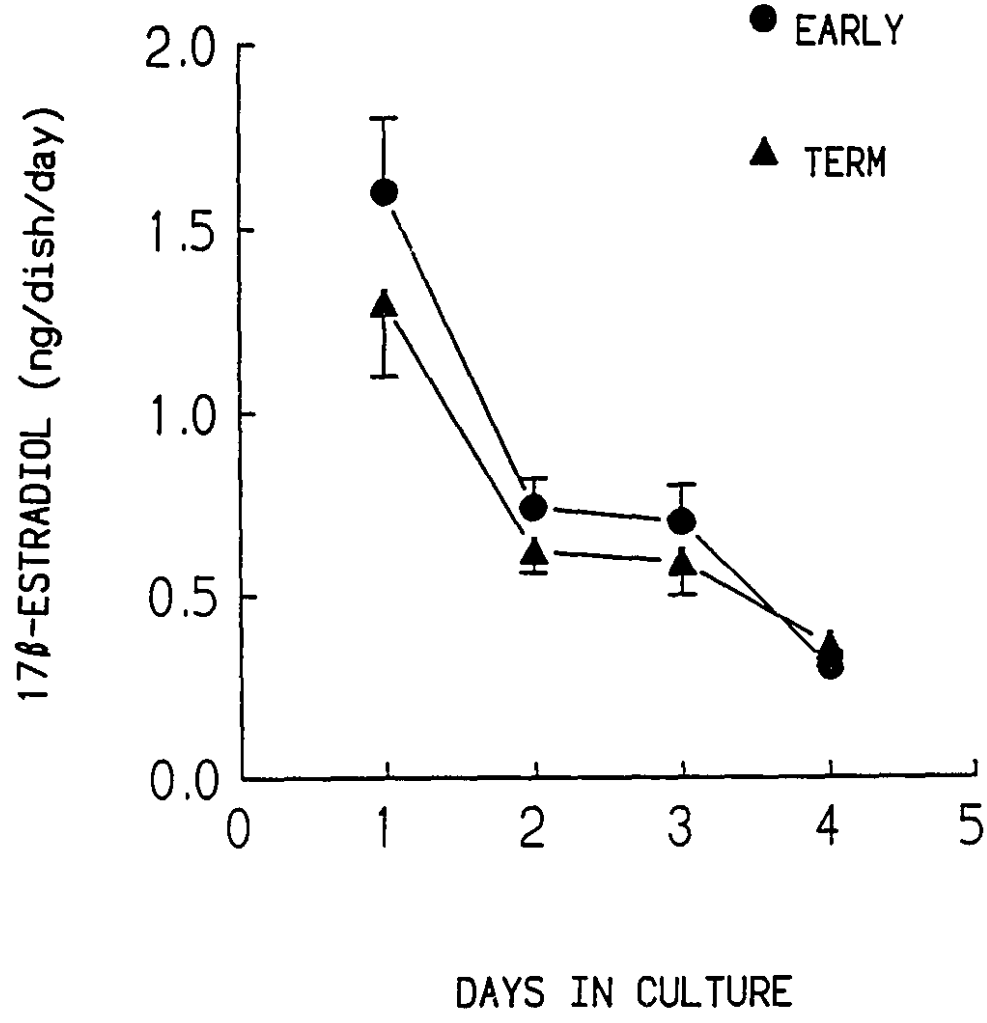


Fig. 6.3 Basal estradiol production from early and term placental culture with Ham's F-10+FBS 10%. Each data point is the mean ± SE of quadruplicate observations (duplicate determinations for each of two placentas).

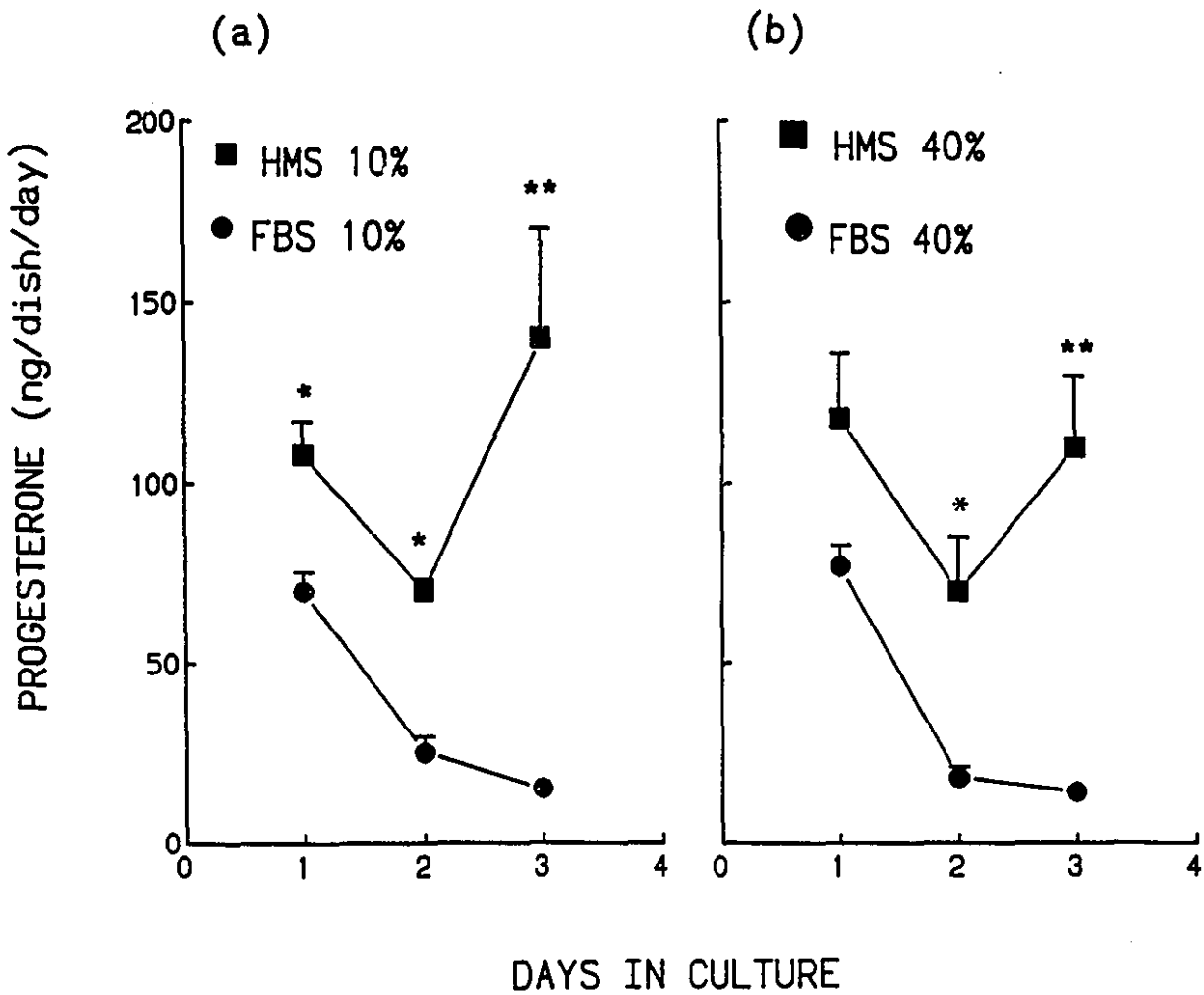


Fig. 6.4 Comparison of basal progesterone production by early placenta in presence of 10 or 40% fetal bovine serum (FBS) and 10 or 40% human maternal serum (HMS). Results are the mean ± SE of quadruplicate observations. * = $p < 0.05$; ** = $p \leq 0.01$ compared to that in FBS on the same day. Two different placentas were determined in duplicate for each point.

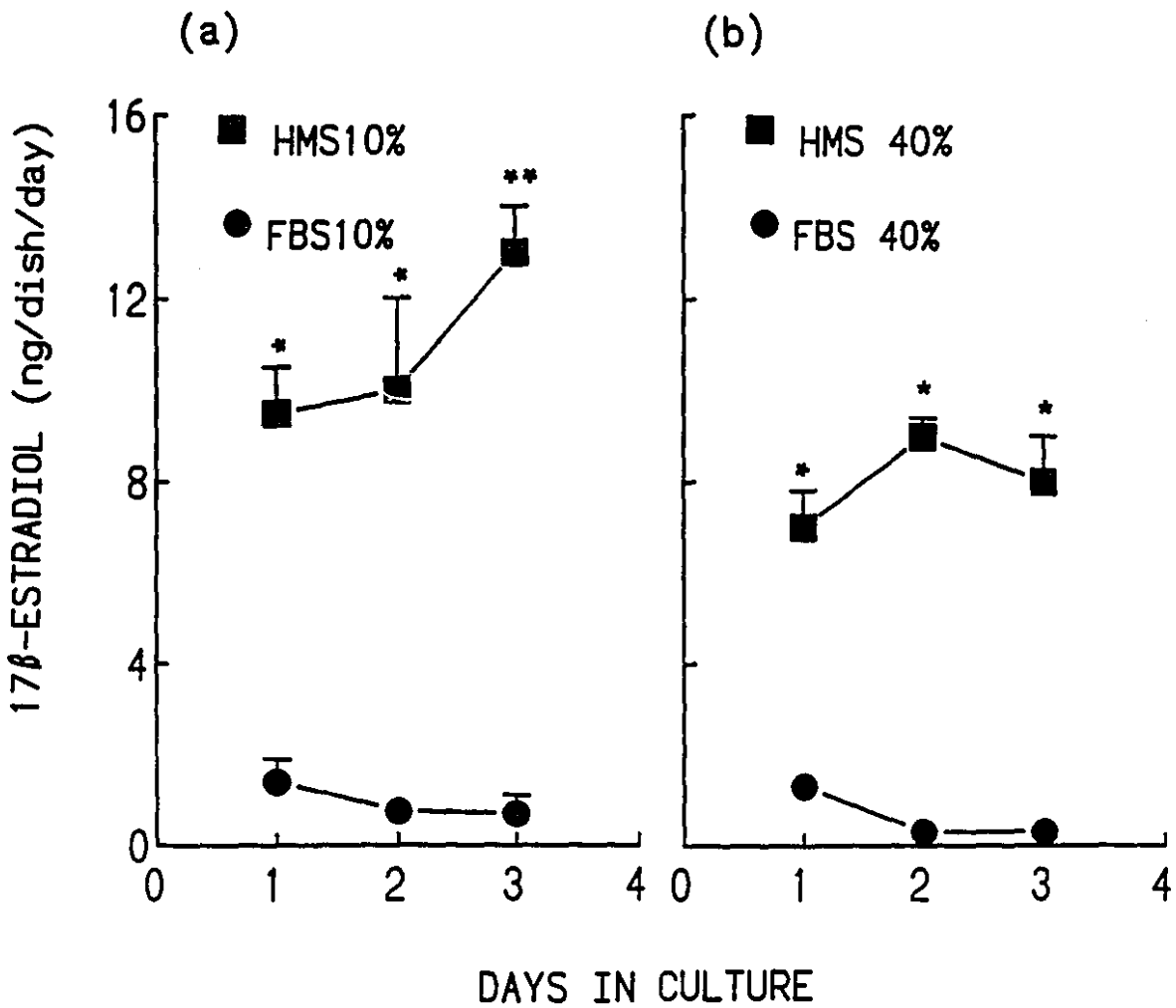


Fig. 6.5 Comparison of the estradiol production by early placenta in presence of 10 or 40% fetal bovine serum (FBS) and 10 or 40% human maternal serum (HMS). Values are the mean ± SE of quadruplicate observations. * = $p<0.05$; ** = $p\leq 0.01$ compared to that in FBS. Two different placentas were used.

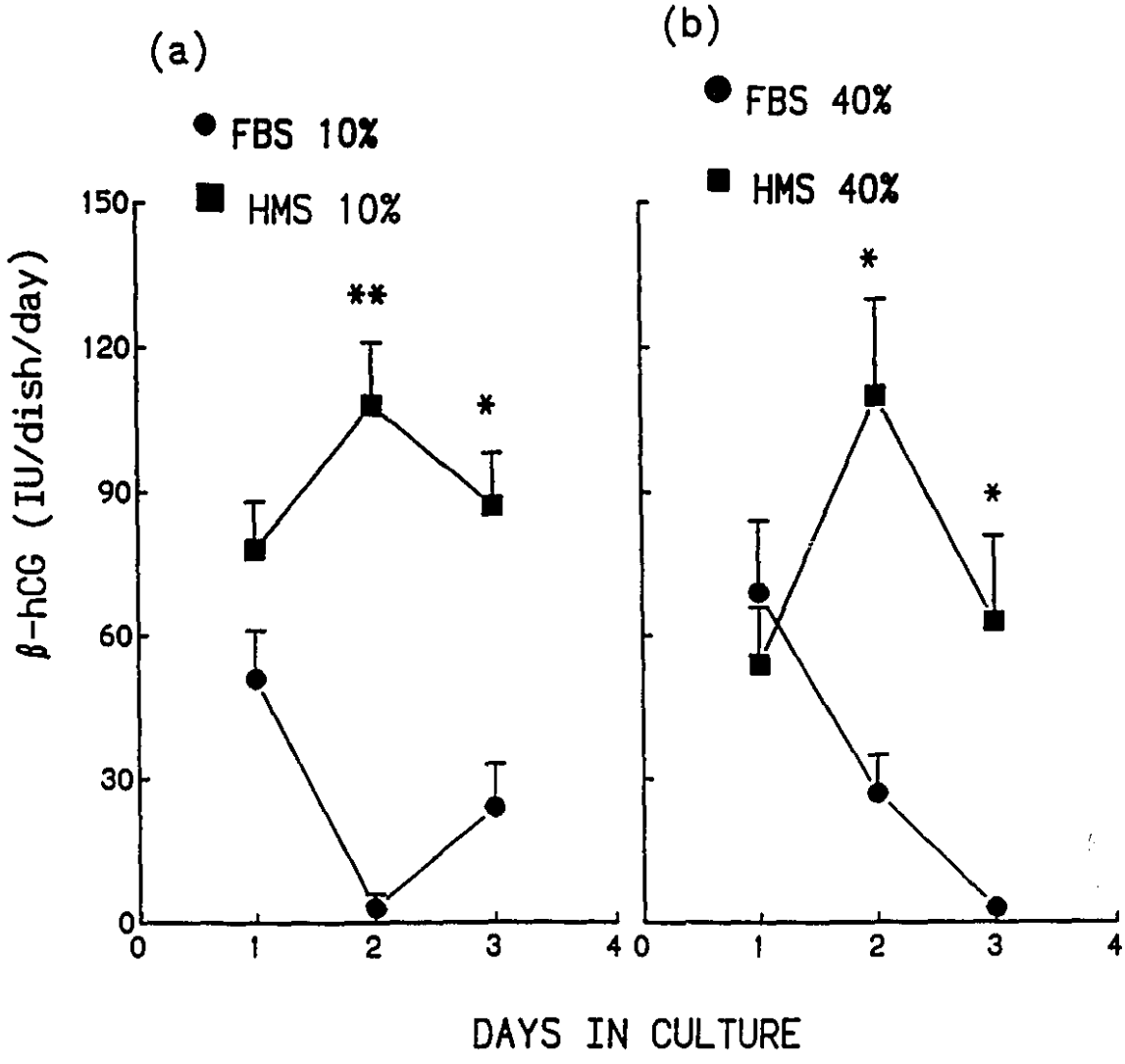


Fig. 6.6 The production of early placental human chorionic gonadotropin (β-hCG) in presence of 10 or 40% human maternal serum (HMS) and 10 or 40% fetal bovine serum(FBS). Each data point is the mean ± SE of quadruplicate observations. * = $p < 0.05$, ** = $p < 0.01$ compared to that in FBS for respective days. Two different placentas were used.

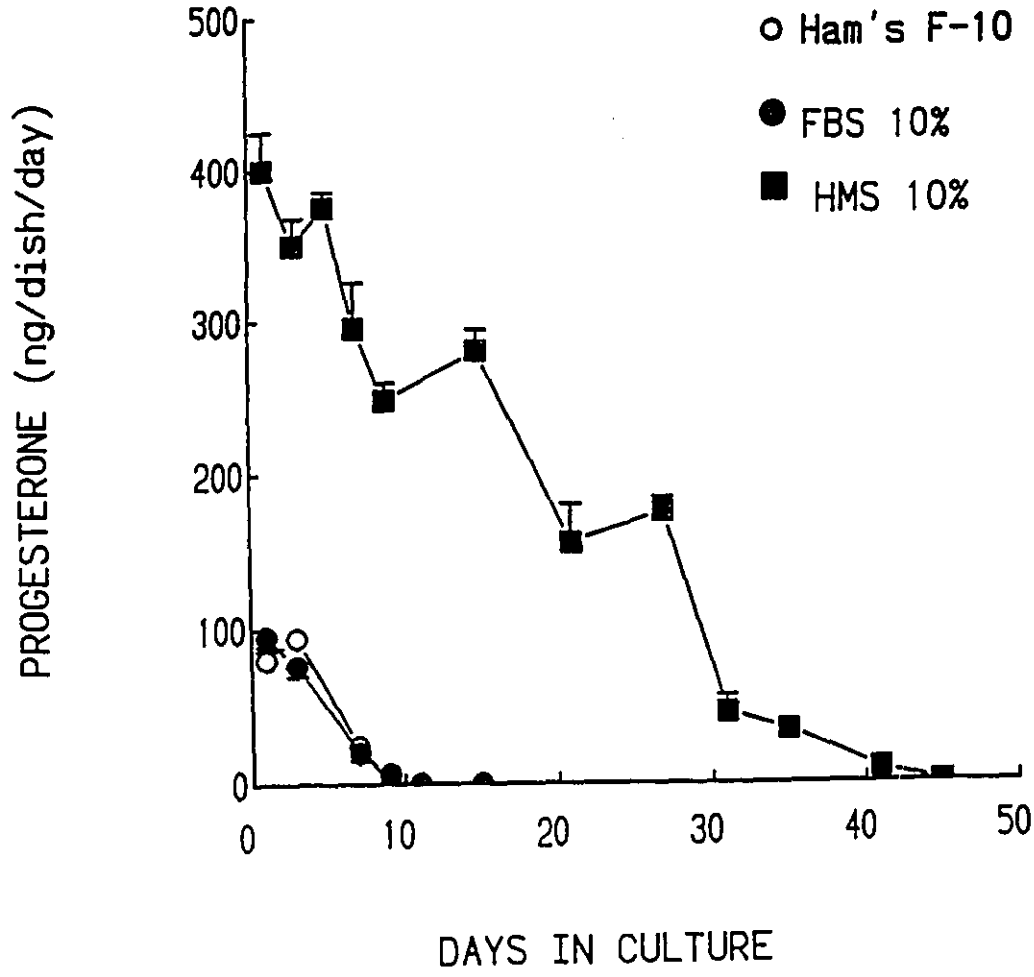


Fig. 6.7a Early placental progesterone production in the presence of FBS 10% or HMS 10% or in the absence of serum (Ham's F-10). Each point represents mean ± SE of quadruplicate observations from a single placenta.

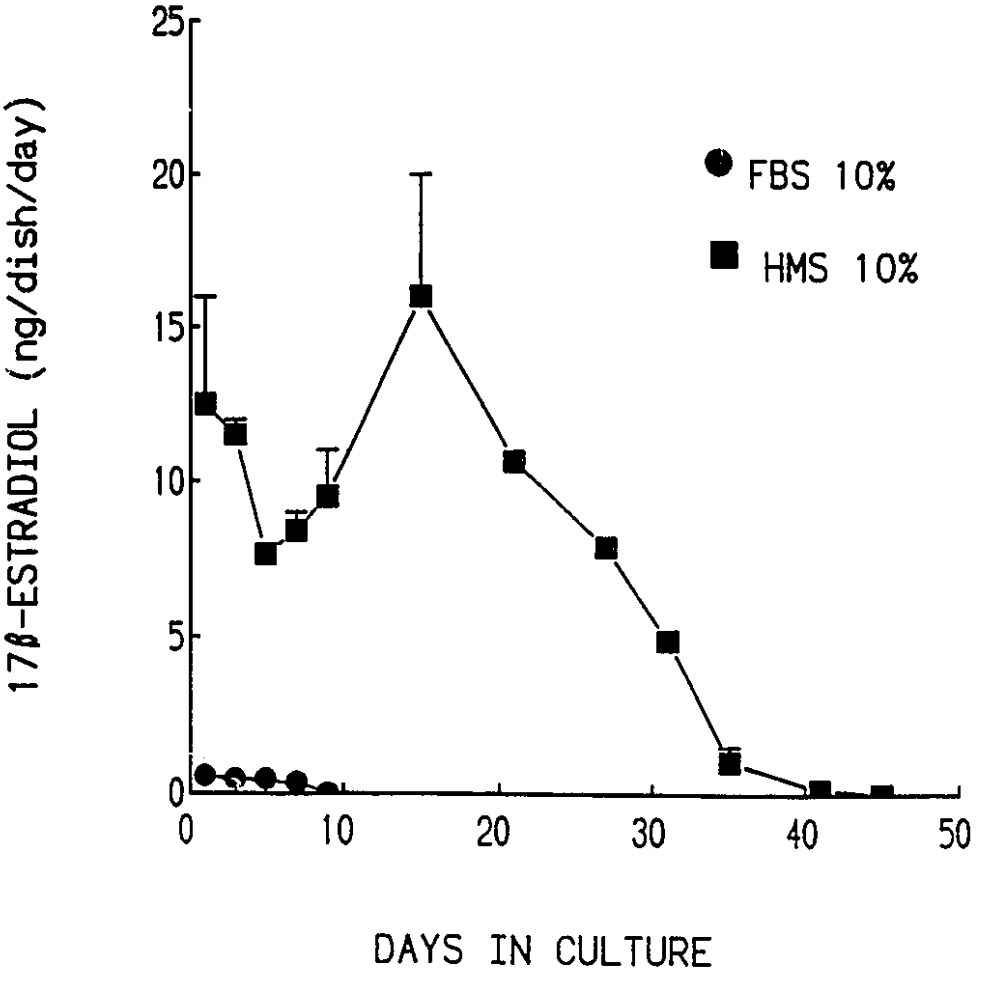


Fig. 6.7b Early placental estradiol production in the presence of FBS 10% or HMS 10%. Each point represents mean ± SE of quadruplicate observations from a single placenta.

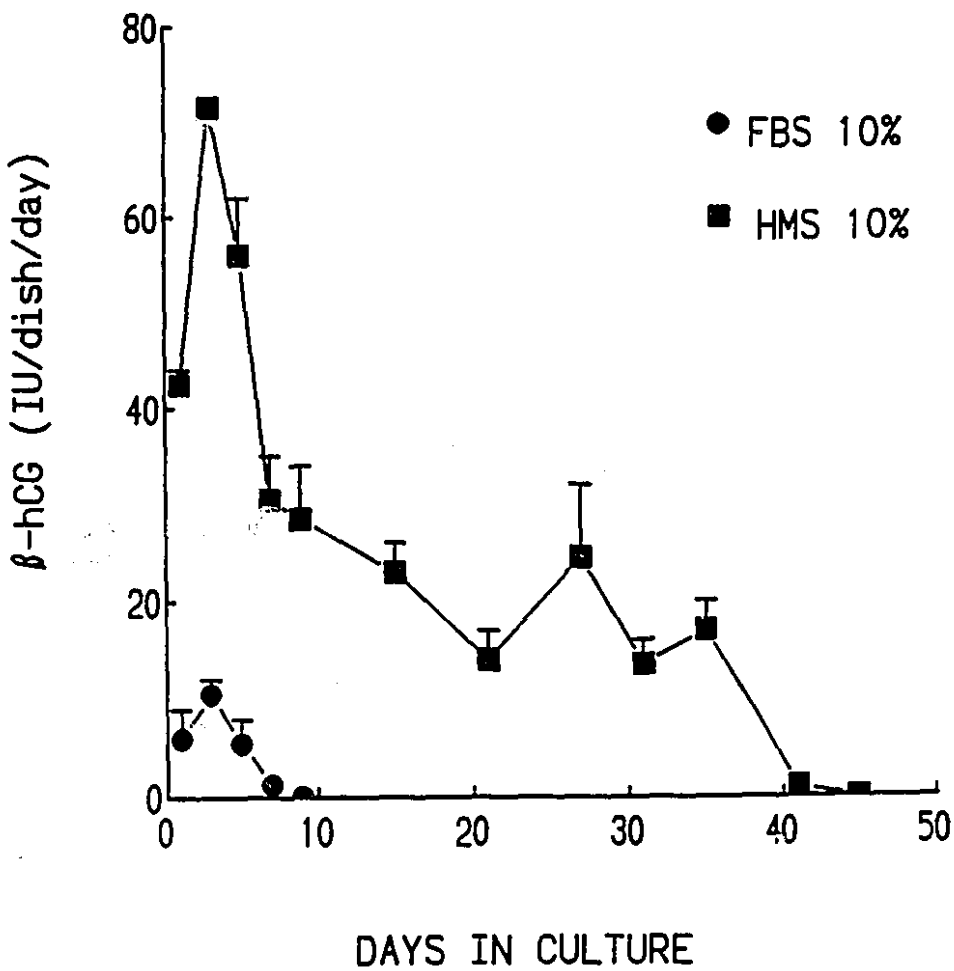


Fig. 6.7c Early placental ß-hCG production in the presence of FBS 10% or HMS 10%. Each point represents mean ± SE of quadruplicate observations from a single placenta.

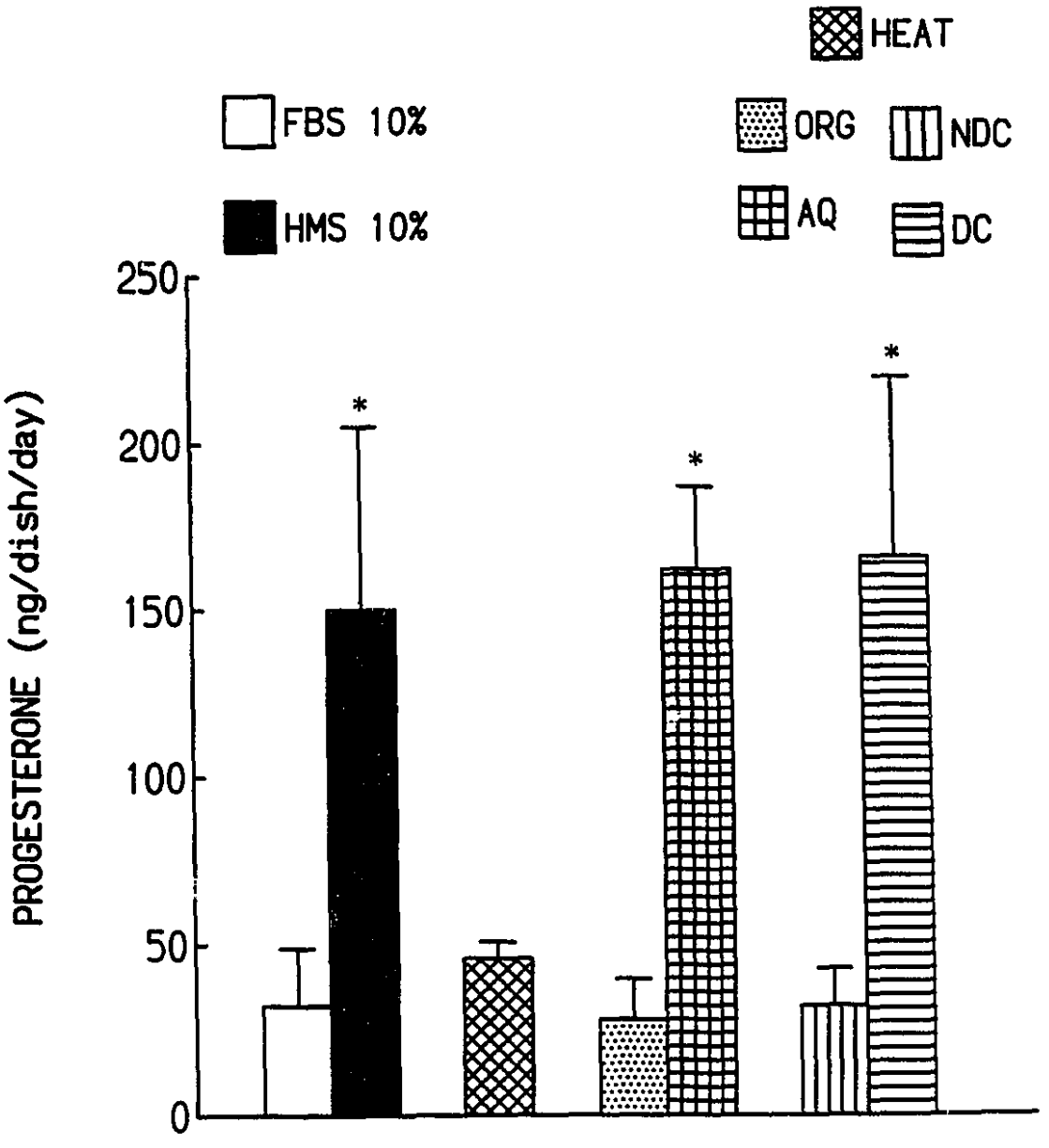


Fig. 6.8 Progesterone production by early placenta in the presence of treated human maternal serum (10%). Heat = HMS heated at 60^0C for one hour; ORG = HMS extracted into diethyl ether, AQ = aqueous phase after extraction; DC = dialyzed component, NDC = non dialyzed component. Each bar represents mean ± SE of quadruplicate observations. * = $P \leq 0.05$ compared to that in FBS.

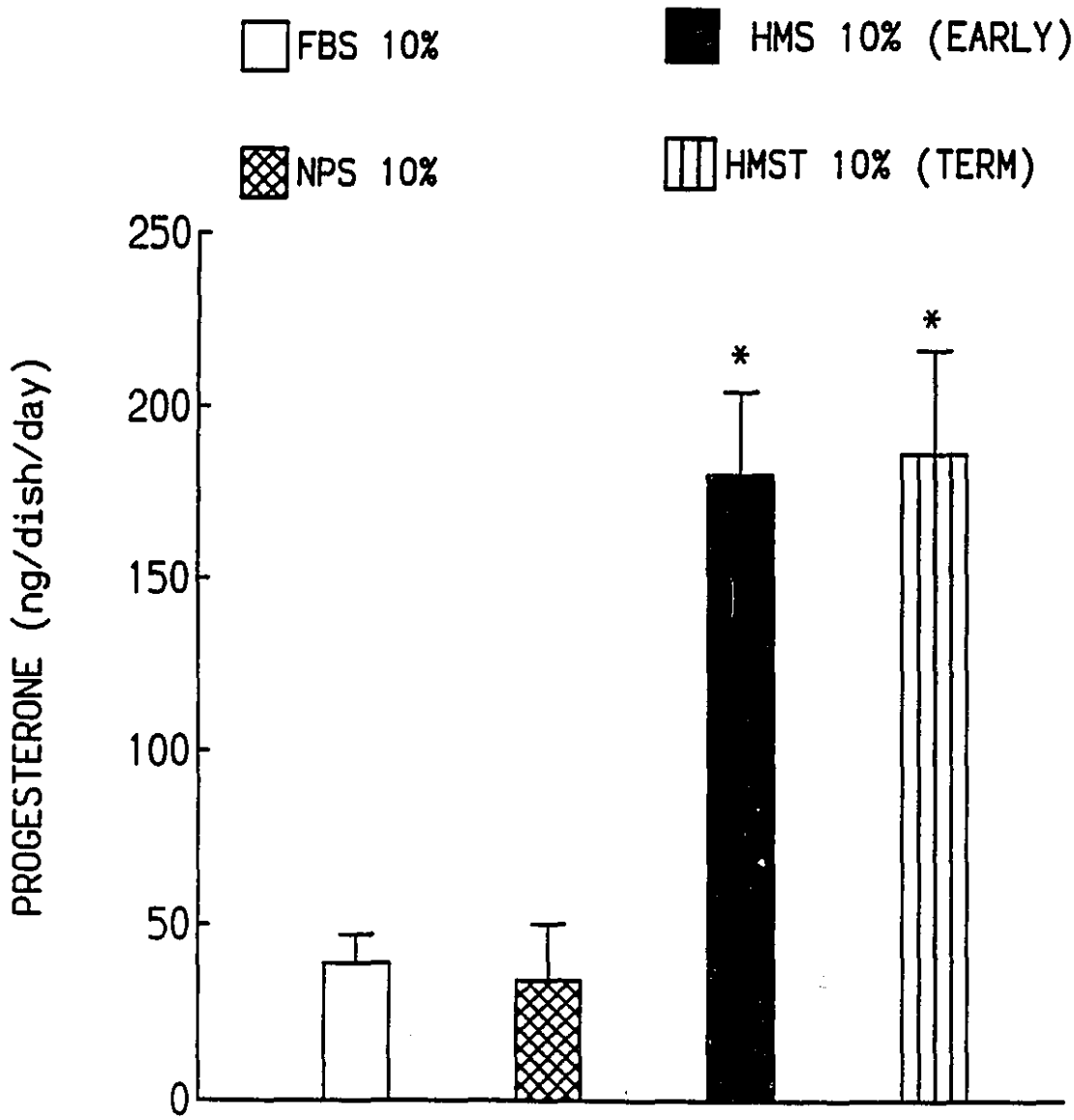


Fig. 6.9 Progesterone production by early placenta in presence of 10% FBS, non pregnant and pregnant human maternal serum from early as well as from late gestation. Each bar represents mean ± SE of quadruplicate observations from a single placenta. * = $P \leq 0.05$ compared to that in FBS.

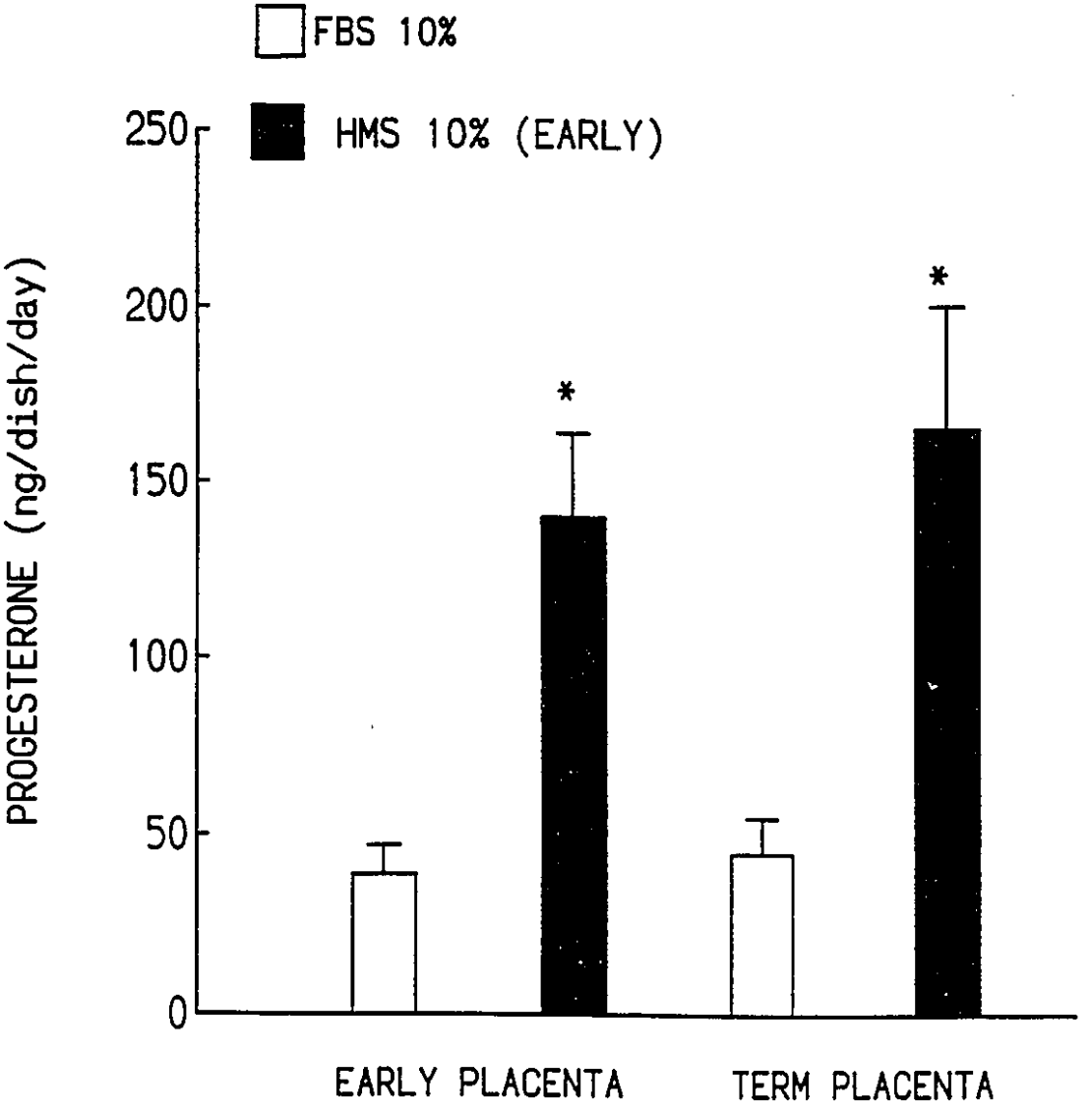


Fig. 6.10 Progesterone production from early and term placental culture in presence of 10% FBS or HMS 10%. Each bar represents mean ± SE of quadruplicate observations. * = $P \leq 0.05$ compared to that in FBS.

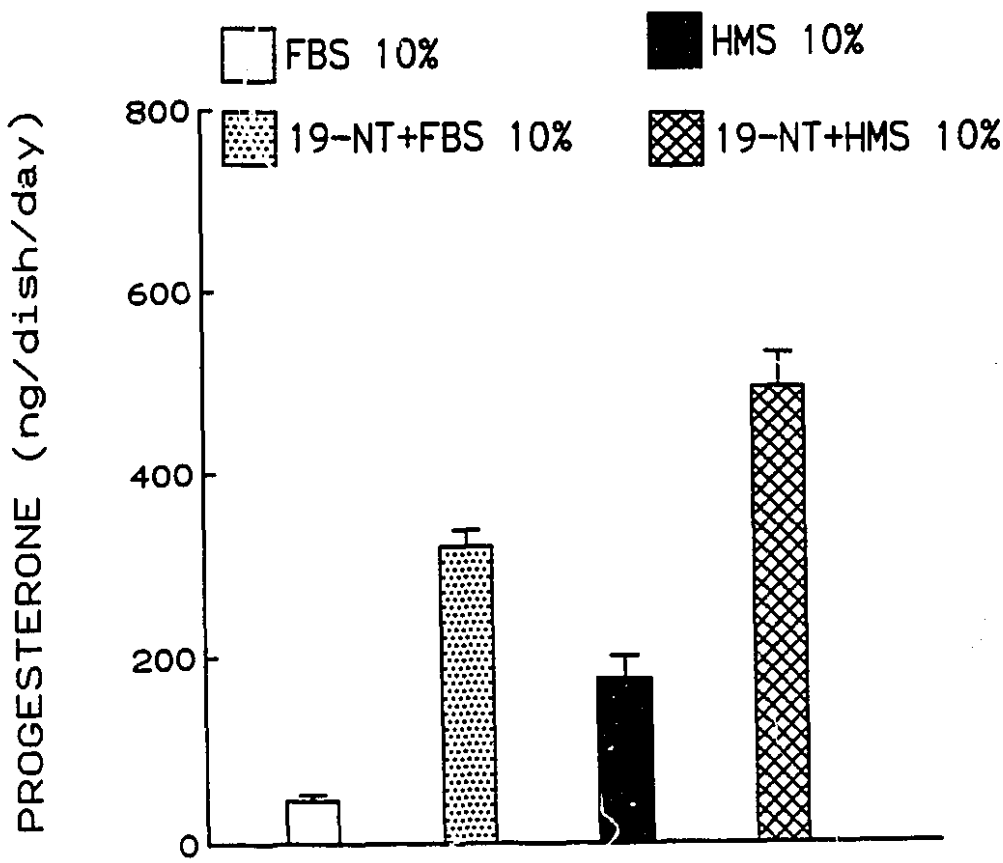


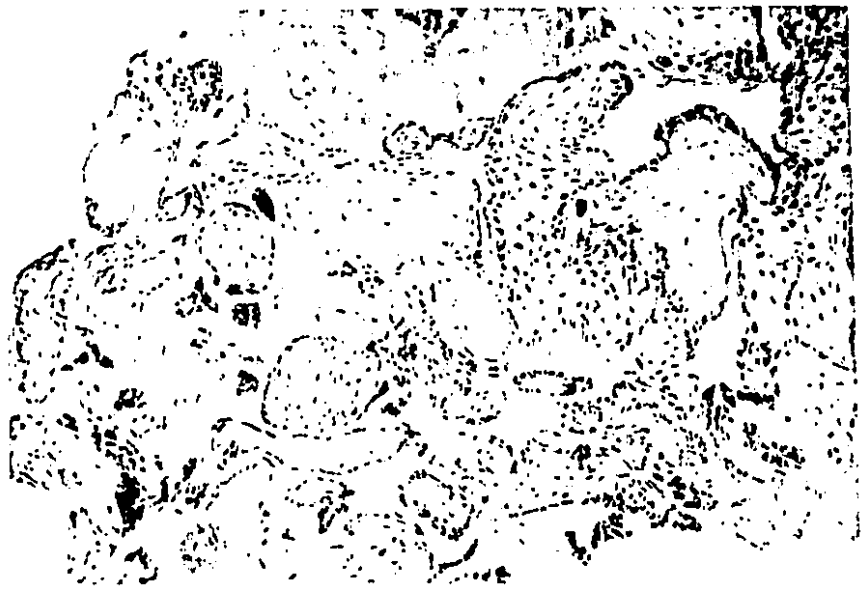
Fig. 6.11 Progesterone production by early placenta in the presence of 10% FBS, 10% HMS, 1 μM 19-nortestosterone (19-NT) + 10% FBS, or 19-NT + 10% HMS. Each bar represents the mean ± SE of quadruplicate determinations. The differences of each group from each of the others was highly significant ($P \leq 0.001$).

a)

b)

Fig. 6.12: Fresh placental tissue before culture, from first trimester human pregnancy, demonstrating the tissue morphology of placental villi. H/E stain. a) 100x magnification; b) 250x magnification.

FBS 10%



HMS 10%

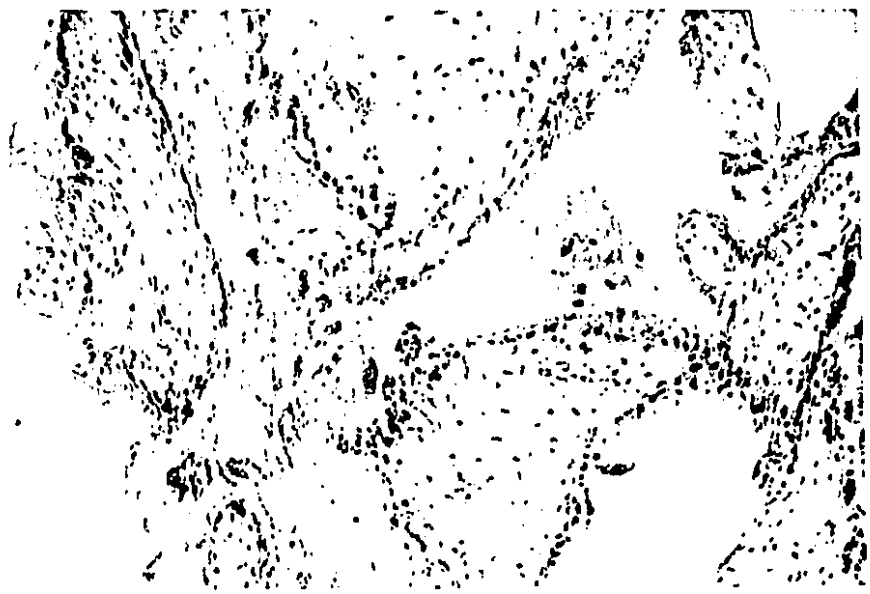


Fig. 6.13: Viability of early placental explants after 24 hours in culture under different conditions. The tissue morphology is well maintained and there is no evidence of focal necrosis in the presence of foetal bovine serum (FBS 10%) and early human maternal serum (HMS 10%). (H/E stain, 100x magnification)

FBS 10%

HMS 10%

Fig. 6.14: Viability of early placental explants after seven days in culture under different conditions. While placental explants in the presence of foetal bovine serum (FBS 10%) are necrotic, villous morphology is well maintained in the presence of early human maternal serum (HMS 10%). (H/E stain, 100x magnification)

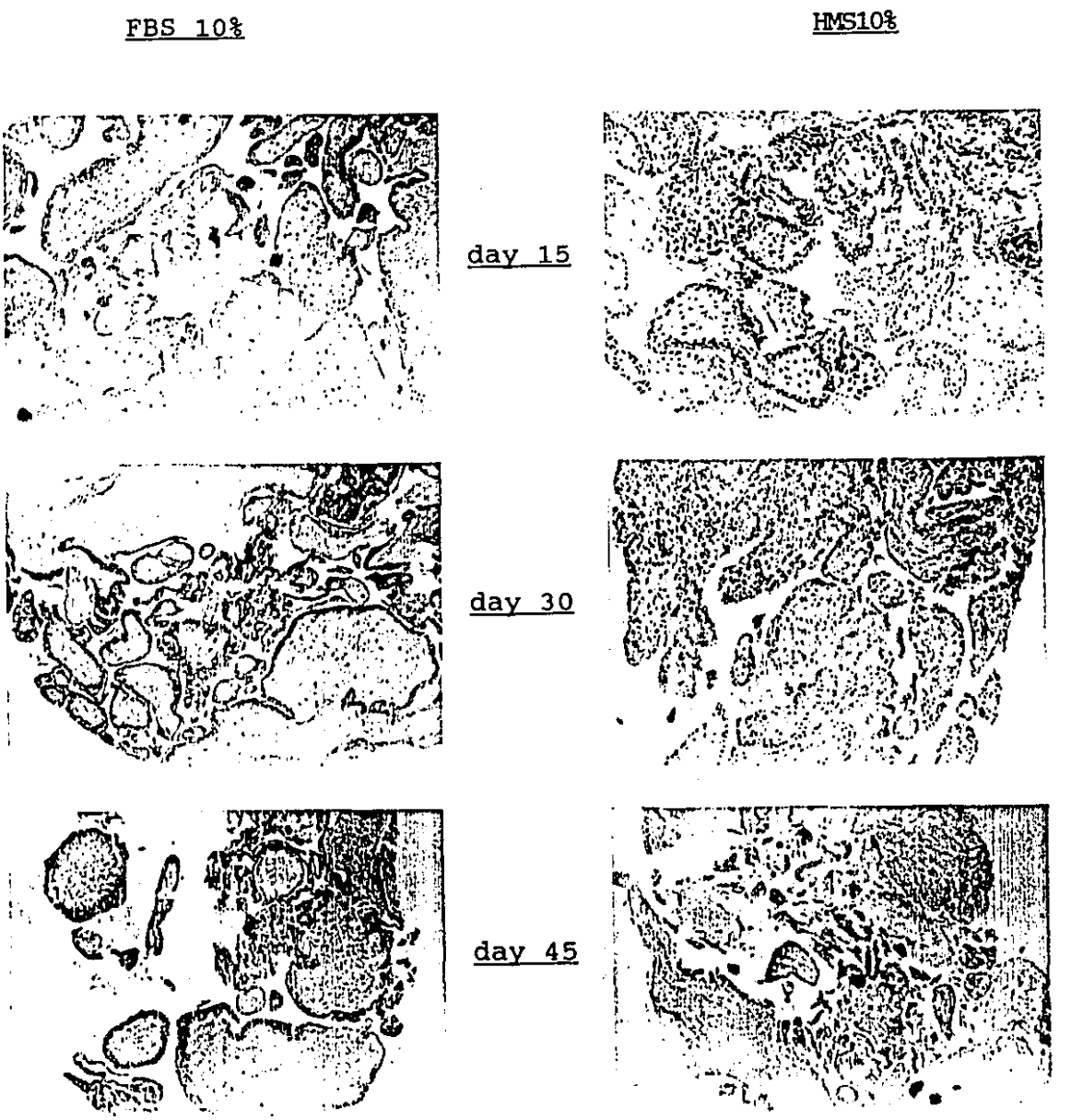


Fig. 6.15: Viability of early placental explants on different days in culture under different conditions. While explants in the presence of foetal bovine serum (FBS 10%) (left column) are necrotic, in the presence of early human maternal serum (HMS 10%) (right column) explants remain viable until day 30, and are necrotic by day 45. (H/E stain, 100x magnification)

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CHAPTER VII

CLOSURE

7.1 SUMMARY AND CONCLUSIONS

Little is known about the factors regulating placental progesterone production at the time of the luteo-placental shift. To explore the regulatory mechanism, the effects of various steroids and peptides on the production of progesterone by placental explants obtained from early gestation (6-10 weeks) were studied. Relevant comparative studies were undertaken with placental samples from normal pregnancy at term.

In summary it was found that In early placental explant cultures, progesterone production was stimulated by 19-nortestosterone (19-NT) and by some androgens, namely, androstenedione (A-dione), 5α-androstane-3α,17ß diol (3α-diol) and 5α-androstane-3ß,17ß diol (3ß-diol). Of all the compounds tested, 19-NT had the greatest effect, A-dione had intermediate effects, and the 5α-reduced steroids (3α- and 3ß-diol) caused only slight increases.

Along with other steroids (testosterone, dehydroepiandrosterone sulfate, dexamethasone) estrogens had no influence on placental progesterone production. These observations suggest that progesterone stimulation by androgens and a norandrogen is not mediated through estrogens; instead a direct action may

be involved. The observed lack of effect of cAMP on progesterone production indicates the unlikelihood of adrenergic action as a mediator of progesterone stimulation in our system. The putative tropic hormones (hCG, GnRH, ACTH) were also found to be ineffective.

The stimulatory effects of 19-NT and androgens (except 3ß-diol) on early placental progesterone production were not observed in the comparative studies with term placenta.

It was found that the ability of androgens and the norandrogen to stimulate progesterone secretion was inversely proportional to their capability of being aromatized. The 19-NT was the least effective of those studied. The efficiency of aromatization for A-dione was also much lower than that for another methyl androgen (Δ^5-Androstene-diol). Since 19-NT and A-dione were poorly aromatized, they may well be present in sufficient concentrations in early placenta to be important regulators of progesterone production.

To know the physiological significance of progesterone stimulation by the above mentioned androgens and the norandrogen, their concentrations were measured in placenta and confirmed their presence in substantial amounts. The detection of 19-NT in pregnancy plasma also suggested its origin in placenta (Reznik et al., 1987). The higher concentrations of placental 19-NT and A-dione in early pregnancy compared to those at term are in accord with the observations of their stimulatory influences on progesterone production at the same

period of gestation. Consistent with the observed progesterone stimulation by 3ß-diol in both early and late placental cultures, placental levels of 5α-reduced steroids rose progressively from early to late gestation.

The mechanism of the progesterone stimulation was studied to determine whether it was due to increased synthesis or decreased catabolism of placental progesterone. The lack of effect on progesterone metabolism in early placenta suggested that the stimulation involves biosynthesis.

It was interesting to note that the principal enzymes involved in progesterone biosynthesis (P450scc and 3ß-HSD) were influenced by 19-NT. This result suggests that the progesterone stimulation by 19-NT is mediated by the combined effects on both the synthetic enzymes. Since A-dione and 3α-diol increased placental P450scc while 3ß-HSD activity was increased by 3ß-diol, their stimulatory influences on progesterone production observed were due to their effects on the respective biosynthetic enzymes.

To determine whether the above progesterone stimulations were associated with protein synthesis, investigations with cycloheximide (CH) were carrried out. The concurrent CH treatment of early placental cultures decreased the progesterone stimulation by all four steroids. This result suggested that progesterone stimulation by androgens and 19-NT required new protein synthesis. However, the initial phase of 19-NT- stimulated progesterone production within two hours was

not blocked by the treatment of CH. This study suggests an additional involvement of a CH-non-inhibitable process along with a CH-inhibitable process, in 19-NT- stimulated progesterone production.

To improve placental basal in vitro hormone production, the use of human maternal serum (HMS) in place of fetal bovine serum (FBS), as a supplement to the culture medium (Ham's F-10) was studied. It was found that the production of progesterone as well as estradiol and β-hCG were improved in the presence of HMS. The above observation was confirmed both histologically and biochemically; there was a good correlation ($n = 0.74$, $P < 0.05$) between these two parameters. The characterization of the stimulatory activity of HMS on progesterone production showed that it was heat labile, and not extractable into organic solvent or dialyzable. These observations suggest that the stimulatory influence of HMS on placental progesterone production is protein in nature. The prolongation of the viability of placental culture to 30 days indicates the importance of circulatory protein in pregnancy serum for placental development as well as for endocrine functions.

Progesterone stimulation by 19-NT (1 μM) in culture with FBS 10%, was about two fold higher than that by HMS 10% alone. The combined stimulatory effects of 19-NT and HMS 10% on progesterone production were additive, suggesting that stimulation of progesterone production by 19-NT and HMS were

not through a common mechanism.

In conclusion, this study suggests that 19-NT along with certain androgens is important for the regulation of placental progesterone secretion at the time of the luteo-placental shift. For in vitro study of placental hormonal regulation, human maternal serum is a better supplement economically as well as physiologically.

7.2 Physiological Implications of Progesterone Stimulation

The importance of placental progesterone production for the maintenance of pregnancy is well documented in the literature. In this study, progesterone regulation by androgens and a norandrogen suggests an important role of C_{18} and C_{19} steroids in the maintenance of pregnancy.

Since the endocrine function of placental progesterone is important both in early as well as in late pregnancy, it is important to understand the relationship between placental progesterone with its surrounding hormones. The presence of 19-NT and A-dione along with progesterone in the trophoblast supports the observed stimulatory influences of 19-NT and A-dione on placental progesterone production. In accord with my observation in case of 19-NT and A-dione, placenta obtained from the early part of gestation has been shown to have larger potential for the synthesis of hormones such as hCG (Diczfaulsy, 1953; Ahmed et al., 1990) and estradienolone - a newly

discovered norsteroid (Philip and Murphy, 1989a,b).

The natural occurrence of 19-norsteroids and their possible functional importance are now more generally accepted than previously thought. For instance, the presence of 19-nor-deoxycorticosterone and 19-norcorticosterone in rat and man (Dole et al., 1981, 1985; Gomez et al., 1979) and their high potency in a sodium retaining bioassay (Kagawa et al., 1957) were reported earlier. Fiser et al. (1959) showed that 19-NT has higher anabolic activity and norethisterone has higher potency as an oral progesterone than their respective parent compounds.

Philip (1986) hypothesized that the biological importance of the presence of a norsteroid - an estradienolone, in plasma, is associated with the maintenance of pregnancy and its decrease with the initiation of labor. Since placental concentrations of both the norsteroids (19-NT and estradienolone) are high in the early part of gestation and fall by term, both might have functional similarities in terms of pregnancy maintenance. Therefore, like estradienelone in Philip's study (1986), it is hypothesized from the present study that 19-NT is involved in the hormonal support of early pregnancy by its enhancing capacity of progesterone production.

Although the decline of circulatory progesterone level associated with labor has not been proved in human, the preliminary data from our laboratory (Ahmed, 1990) suggests that the placental progesterone concentration declines at

term. If this finding is confirmed, it would agree with my speculation that the decreased level of 19-NT in placenta is associated with the precipitation of labor by the lack of influence of 19-NT on progesterone production. The availability of progesterone and its stimulatory steroids in the same tissue of trophoblast suggest an auto or paracrine control. As mentioned earlier (Reznik et al., 1987), the detection of 19-NT in pregnancy serum and the authors' suggestion of its placental origin supports the above hypothesis.

Knowledge of the mechanism of the stimulation of progesterone production by 19-NT might have an impact on the development of better means for the prevention of early loss of pregnancy which most often occurs at or just after the time of the luteo-placental shift. The knowledge of progesterone regulation by 19-NT could further lead to the discovery of more effective means of contraception or less invasive means of pregnancy termination.

7.3 Recommendations for Future Work

1. An investigation into the molecular basis of progesterone stimulation by 19-nortestosterone and its effect on the expression of progesterone biosynthetic enzymes along with the enzyme concentrations should be of interest.

2. The effect of 19-nortestosterone on progesterone production can be studied in a site other than the placenta, particularly in the ovary.

3. An extension of the present study in other primates whose placentas are functionally similar to that of a human placenta should be interesting.

4. To know the exact nature of the protein associated with stimulatory influence of human maternal serum on progesterone production, further characterization of its protein component is necessary.

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CHAPTER VIII

CONTRIBUTIONS TO KNOWLEDGE

1. To the best of my knowledge this is the first study of progesterone regulation in the human placenta obtained in early gestation.

   i) In this study, 19-nortestosterone and certain androgens (androstenedione, 5α-androstane-3α,17β diol and 5α-androstane-3β,17β diol) stimulated progesterone production.

   ii) Estrogens, dehydroepiandrosterone and its sulfate and other steroids (testosterone, cortisol) and peptides (β-hCG, GnRH, ACTH) were without effect.

2. In term placenta, only 5α-androstane-3β,17β diol stimulated progesterone production; 19-nortestosterone, androstenedione and 5α-androstane-3α,17β diol, did not have any effect.

3. This is the first report of the concentrations of 19-nortestosterone, androstenedione and 5α-reduced steroids in human placenta.

   i) The levels of 19-nortestosterone and androstenedione were high in early but were low in late placenta.

   ii) The 5α-reduced steroids (3α- and 3β-diol) in

placenta increaseed with the length of gestation.

4. Placental biosynthetic enzymes are influenced by 19-norandrogen and androgens in early pregnancy.

i) 19-nortestosterone stimulates both steps of progesterone biosynthesis: P450scc and 3ß-HSD enzyme activities.

ii) Both A-dione and 3α-diol stimulated P450scc while 3ß-diol increased 3ß-HSD activity.

5. The stimulatory influences of all four steroids on progesterone were blocked by cycloheximide.

6. It was shown for the first time that early placental basal hormone productions (progesterone, estradiol, ß-hCG) were improved and prolonged in the presence of human maternal serum.

i) The above observations were confirmed both biochemically and histologically.

7. The stimulatory influence of human maternal serum was found to be protein in nature.

APPENDIX 1: POWDER FORMULATION of HAM'S F-10

The following formulation of Ham's F-10 nutrient mixture is taken from the Grand Island Biological Co., (Burlington, Ontario), catalogue 1989/1990.

Inorganic salts	mg/L
$CaCl_2.2H_2O$	33.29
$CuSO_4.5H_2O$	0.0025
$FeSO_4.7H_2O$	0.834
KCL	285.0
KH_2PO_4	83.0
$MgSO_4$ (anhydrous)	74.64
NaCl	7400.0
Na_2HPO_4 (anhydrous)	153.7
$ZnSO_4.7H_2O$	0.0288
Other components	
Glucose	1100.0
Hypoxanthine	4.68
Lipoic acid	0.2
Phenol red	1.2
Sodium pyruvate	110.0
Thymidine	0.7
Amino acids	
L-alanine	9.0
L-arginine HCl	211.0
L-asparagine.H_2O	15.01
L-aspartic acid	13.0
L-cysteine	25.0
L-glutamic acid	14.7
L-glutamine	46.0
Glycine	7.51
L-histidine HCl.H_2O	23.0
L-isoleucine	2.6
L-leucine	13.0
L-lysine HCl	29.0
L-methionine	4.48
L-phenylalanine	5.0
L-proline	11.5
L-serine	10.5
L-threonine	3.57
L-tryptophan	0.6
L-tyrosine (Disodium salt)	2.62
L-valine	3.5
Vitamins	
Biotin	0.024
D-Ca pantothenate	0.715
Choline chloride	0.698

Vitamins	mg/L
Folic acid	1.320
i-Inositol	0.541
Niacinamide	0.615
Pyridoxine HCl	0.206
Riboflavin	0.376
Thiamine HCl	1.000
Vitamin B_{12}	1.360

$NaHCO_3$, 1200 mg/L, was added to the medium following the addition of distilled water.

APPENDIX 2 : SPECIFICITY OF GUINEA PIG TRANSPROGESTIN[1]

TRIVIAL NAME	SYSTEMATIC NAME	% CROSS-REACTIVITY
Progesterone (P)	Pregn-4-ene-3,20-dione	100
Medrogestone	6,17 ν-Dimethylpregn-4,6-diene-3,20-dione	350
5α-Dihydro-P	5α-Pregnane-3,20-dione	65
5β-Dihydro-P	5β-Pregnane-3,20-dione	30
20α-Dihydro-P	20α-Hydroxypregn-4-ene-3-one	37
20β-Dihydro-P	20β-Hydroxypregn-4-ene-3-one	6
Desoxycorticosterone	21-Hydroxypregn-4-ene-3,20-dione	43
11-Keto-P	Pregn-4-ene-3,11,20-trione	1.65
11β-OH-P	11β-Hydroxypregn-4-ene-3,20-dione	0.95
17α-OH-P	17α-Hydroxypregn-4-ene-3,20-dione	0.06
16-Dehydro-P	Pregn-4,16-diene-3,20-dione	2.1
18-OH-P	18-Hydroxypregn-4-ene-3,20-dione	5.5
Provera	6α-Methylpregn-4-ene-3,20-dione-17α-yl	1.35
11-Desoxy-cortisol	17α,21-Dihydroxypregn-4-ene-3,20-dione	0.9
Pregnenolone	3β-Hydroxypregn-5-ene-20-one	2.3
Testosterone (T)	17β-Hydroxyandrost-4-ene-3-one	8.0
1-Dehydro-T	17β-Hydroxyandrost-1,4-diene-3-one	0.7
Dihydrotestosterone (DHT)	17β-Hydroxy-5α-androstan-3-one	20
7αCH3-DHT	17β-Hydroxy-7α-methyl-5α-androstan-3-one	0.5
17αCH3-DHT	17β-Hydroxy-17α-methyl-5α-androstan-3-one	26
7α,17α-Dimethyl-DHT	17β-Hydroxy-7α-17α-dimethyl-5α-androstan-3-one	2.2
9α-Flouro-11βOH-17αCH3-DHT	9α-Flouro-11β,17α-dihydroxy-17α-methyl-5α-androstan-3-one	3.6
Androstenediol	Androst-4-ene-3β,17β-diol	0.5
	5α-Androstan-3,17-dione	12
	5β-Androstan-3,17-dione	0.65
Androstenedione	Androst-4-ene-3,17-dione	2.0
	Androst-1,4-diene-3,17-dione	0.5
	5α-Androstane-3β-ol	1.0
19-Nor-testosterone (19-nor-T)	17β-Hydroxyestr-4-ene-3-one	17
2α-Methyl-19-nor-T	17β-Hydroxy-2α-methylestr-4-ene-3-one	13
4-Methyl-19-nor-T	17β-Hydroxy-4-methylestr-4-ene-3-one	53
7α-Methyl-19-nor-T	17β-Hydroxy-7α-methylestr-4-ene-3-one	2.1
7β-Methyl-19-nor-T	17β-Hydroxy-7β-methylestr-4-ene-3-one	46
17α-Methyl-19-nor-T	17β-Hydroxy-17α-methlyestr-4-ene-3-one	26
7α,17α-Dimethyl-19-nor-T	17β-Hydroxy-7α,17α-dimethylestr-4-ene-3-one	15
17α-Ethinyl-7α-methyl-19-nor-T	17β-Hydroxy-17α-ethinyl-7α-methylestr-4-ene-3-one	32
6-Dehydro-19-nor-T	17β-Hydroxyestr-4,6-diene-3-one	19
19-Nor-DHT	17β-Hydroxy-5α-estran-3-one	29

Appendix 2 contd...

TRIVIAL NAME	SYSTEMATIC NAME	% CROSS-REACTIVITY
7α-Methyl-19-nor-DHT	17β-Hydroxy-7α-methyl-5α-estran-3-one	1.2
7α,17α-Dimethyl-19-nor-DHT	17β-Hydroxy-7α,17α-dimethyl-5α-estran-3-one	3.2
	17β-Hydroxy-4,4-dimethylestr-5-ene-3-one	160

[1] All other tested compounds cross-reacted less than 0.5%. Such compounds included androgens and estrogens.

Data from Tan and Murphy, 1974.

APPENDIX 3

SPECIFICITY OF THE PROGESTERONE ANTISERUM (P11-192)[1]

Steroid	% Cross-Reactivity
Progesterone	100
Aldosterone	< 0.1
Androstenedione	< 0.1
Cortisol	< 0.1
Cortisone	< 0.1
Dehydroepiandrosterone	< 0.1
Dexamethasone	< 0.1
Estradiol	< 0.1
Estriol	< 0.1
17-hydroxypregnenolone	< 0.1
Prednisolone	< 0.1
Pregnanediol	< 0.01
Pregnanetriol	< 0.1
Prednisone	< 0.1
Corticosterone	0.6
17α-hydroxyprogesterone	0.6
11-Desoxycortisol	0.4
4-pregnen-20ß-ol-3-one	1.3
4-pregnen-20α-ol-3-one	0.8
Deoxycorticosterone	3.3
Pregnanolone	< 0.1
Tetrahydrocortisol	< 0.1
Testosterone	< 0.1

[1]Data from Endocrine Sciences Products (Tarzana, CA).

APPENDIX 4

SPECIFICITY OF SEX HORMONE-BINDING GLOBULIN (SHBG)[1]

Steroid	% Cross-Reactivity
Testosterone	100
Androsterone	1.0
Androstenedione	1.4
Dehydroepiandrosterone	2.5
Dihydrotestosterone	300
5-Androstene-3α,17β diol	130
5-Androstene-3β,17β diol	130
5α-Androstane-3α,17β diol	200
5α-Androstane-3β,17β diol	250
4-Androstene-3β,17β diol	200
5-Estrene-3β,17β diol	250
Estrone	4
Estradiol	50

[1]Murphy, BEP: Profiles of Ligands of Sex Hormone Binding Globulin in Human Serum. J. Steroid Biochem., Vol. 31, No. 3, pp. 257-266, 1988.

APPENDIX 5

SPECIFICITY OF THE 19-NORTESTOSTERONE ANTISERUM[1]

Steroid	% Cross-Reactivity
19-Nortestosterone	100
19-Norandrostenedione	80
Progesterone	≤ 1
Pregnenolone	≤ 0.1
5α-pregnanedione	< 1
5β-pregnanedione	< 1
17β-estradiol	< 1
2-methyl-estrone	< 1

[1]Data from Dr. Murphy's laboratory at Montreal General Hospital.

Since 19-NT in this study was measured after chromatographic purification, interference from other compounds is ruled out.

APPENDIX 6

SPECIFICITY OF THE ANDROSTENEDIONE ANTISERUM[1]

Steroid	% Cross-Reactivity
Androst-4-ene-3, 17-dione	100
Testosterone	0.06
5α-Androstane-3, 17-dione	4.00
5α-Androstane-3, 17-dione	2.89
11 β-Hydroxytestosterone	2.75
11-Oxotestosterone	2.29
11-Deoxycortisol	1.19
Androsterone	0.03
Isoandrosterone	0.26
5α-Dihydrotestosterone	0.05
5β-Dihydrostestosterone	0.31
DHA-SO_4	0.003
Etiocholanolone	0.135
DHA	0.044

[1]Data from the information booklet of androstenedione radioimmunoassay Kit (Catalog No. DSL 4200, January 1988).

APPENDIX 7

SPECIFICITY OF THE ESTRADIOL ANTISERUM[1]

Steroid	% Cross-Reactivity
17ß-estradiol	100
estrone	4
estriol	1.8
cortisol	0.05
testosterone	<0.05
androstenedione	<0.05
dehydroepiandrosterone	<0.05

[1]All other tested compounds cross-reacted less than 0.05%. Data from Dr. Murphy's laboratory.

APPENDIX 8

SPECIFICITY OF THE IMMUNOCORP COATED TUBE hCG ASSAY[1]

Protein Hormone	% Cross-Reactivity
hCG	100
FSH	0.525
hGH	ND
hPL	ND
LH	1.20
TSH	0.006

ND= not detectable

[1]Kit (Cat. No. KTSP-4901), IMMUNOCORP (5800 Royalmount, Montreal, Quebec, Canada H4P 1K5).