EFFECTS OF A DOPAMINE AGONIST (CU 32-085) ON RAT LEYDIG CELLS: FUNCTIONAL AND MORPHOLOGICAL STUDIES

by

GHENIMA DIRAMI

A thesis submitted in fulfilment of the conditions

for the degree of Doctor of Philosophy

of the University of London

January 1992

Department of Biochemistry
Royal Free Hospital School of Medicine
London NW3 2PF
England

DEDICATION

To

My Father and Mother

Dr Eric and Mrs Olga Rossdale

My Brothers and Sister

My Nephews and Nieces

All those who support democracy

and

the revival of The Berber language "Tamazirt" in Algeria

ABSTRACT

The dopamine agonist, 8α-aminoergoline (CU 32-085) (DA) developed for use in the treatment of hyperprolactinaemia and Parkinson's disease induces Leydig cell tumours in rats treated for $2\frac{1}{2}$ years. The aim of the present study was to investigate the changes in Leydig cell function and growth at earlier stages of treatment with the DA which may contribute to the formation of the Leydig cell tumours.

Sprague Dawley rats were treated with CU 32-085 (2mg/kg body weight/day) for 1, 5, 12 or 57 weeks. The Leydig cells from rats treated for 5 weeks produced lower levels of testosterone in response to luteinizing hormone (LH) compared with the controls. The defect in steroidogenesis was associated with a decrease in the numbers of LH receptors and cyclic AMP production, a lesion in steroidogenesis at the 17α-hydroxylase and 17-20 lyase, and an increase in aromatase activity.

Histological examination of testis sections from controls and treated rats revealed the presence of atypical sites of Leydig cells surrounded by clusters of macrophages in the testes from the 57 but not the 5 week treated animals. There was 42% and 31% increase in the number of Leydig cells and macrophages respectively, compared with the controls.

A method for separating Leydig cells from macrophages was developed and heterogeneity of the Leydig cells was demonstrated. Preliminary evidence indicates that CU 32-085 may selectively inhibit the function of the Leydig cells with lower sedimentation velocities.

The effect(s) exerted by CU 32-085 on Leydig cells may result from the increase in circulating levels of LH, ACTH/ glucocorticoids and the decrease in prolactin. Evidence for this was obtained from *in vivo* studies with human chorionic gonadotrophin (hCG) and dexamethasone which showed an effect similar to that of CU 32-085. LH-stimulated testosterone production was inhibited in the hCG-treated rats and dexamethasone caused a further decrease.

The development of Leydig cell tumours caused by treatment with CU 32-085 could be due to the changes in testicular levels of testosterone and oestradiol-17ß which may result in changes in factors involved in the control of Leydig cell growth.

It is concluded that the changes in the biochemical properties of the Leydig cells *in vitro* after treatment with CU 32-085 provide a sensitive system for detecting the early effects of this compound on Leydig cell function and growth.

AKNOWLEDGMENTS

I would like first to thank Professor J.A. Lucy for giving me the opportunity to study for a PhD within The Department of Biochemistry at The Royal Free Hospital School of Medicine. I am very grateful to Professor B.A. Cooke, for suggesting this work, his supervision throughout this study, and especially for offering me the scholarship. My thanks are also due to the members of staff of this Department for being friendly and very helpful. Particularly, I am indebted to Richard Preston for drawing the structures of the compounds in Figures 1.2, 1.4, 1.5, and 1.11 and for being always hepful when ever I faced a computing problem. I would also like to thank Dr S. Perkins and Adam S. Nealis for their help with computing. My thanks are also due to Dr D. Fisher, Cristina Delgado, and Farooq Malik for helping me in setting up the coulter counter; Susan Brown and Rita Moran for their constructive suggestions regarding the immunocytochemical and histological techniques and for being very friendly; Jayesh Gor and Vernon Skinner for their technical help and for being very friendly; Dr T. Hallinan and Dr W. Tampion for being always helpful to answer my basic questions in Biochemistry and Physiology; and not forgetting Bernadette O'Reilly, her wonderful warm and friendly Irish smile and her fun sense of humour, made working in this Department a pleasure.

My special thanks are due to Mr D. Hanks and Mr J. Morrisson for their assistance and constructive suggestions; the staff of the Comparative Biology Unit, particularly Mr D. Moore, Mr B. Greenlagh, and Mr M. de Sousa for their help; Dr L. Poulter and his staff at the Department of Immunology for teaching me the Dynabead and immunocytochemistry techniques used for the separation and characterization of macrophages. I would also like to thank the Staff in the Medical illustration Department and the Medical Library at the Royal Free Hospital for all their help.

I would also like to thank all my colleagues, Robert Abayesakara, Jane Aitken, Joseph Anthoniw, Arja Band, Laiq Chauldry, Micheal Choi, Leshia Kurlak, Laura Phipp, and Anthony West for being friendly and helpful. My special thanks are due to Matthew Rose for introducing me to all the new techniques when I started and for his helpful suggestions and advice; Dr Pilar Lopez-Ruiz for all her help and advice and for her moral support especially when I started my writing up.

My special thanks are due to Dr Katja J. Teerds and all the staff in the Department of Cell Biology (University of Utrecht, The Netherlands) without their help the results presented in Chapter 5 would not have been possible. I would also like to thank them for teaching me the technique, for being very helpful and friendly during my stay there, and for preparing the photographs included in this thesis.

I would like to thank Professor A.P.F. Flint and Dr D.R.E. Abayesakara (Institute of Zoology, London) for the assay of $PGF_{2\alpha}$.

I am very grateful to The Overseas Research Student Scheme (UK), Sandoz Ltd (Basle) and The Algerian Government for their financial support. I would also like to thank The Biochemical Society, The Endocrine Society, The Society for the Study of Fertility and the Trust for Research and Education in The Biology of Reproduction for giving me the opportunity to present my work and for providing the necessary funds to attend the various meetings, and NIH (Bethesda, Maryland, USA) for the generous donations of oLH and hCG (CR127).

I would like to express my gratitude to Dr William Taylor (M.Sc. Supervisor at Newcastle University) without his help, advice, encouragements and contacts I would not have had a chance to do my PhD. I would also like to thank him for his major contribution to the improvement of my scientific English and my knowledge in Biochemistry, Physiology and Endocrinology, and for stimulating my interest in Reproductive Endocrinology.

I am very grateful to Moira Winsor (English Language Teacher at Reading University) for her major contribution to the improvement of my spoken and written English and for being very friendly and helpful.

I would like to thank Professor Boukari and Mr Sahili at the Cultural Department (Algerian Embassy, London) for being very helpful when ever I had a problem.

I would like to express my deepest gratitude to Dr Eric and Mrs Olga Rossdale, without them the completion of my thesis would have been impossible. I would like to thank them for their hospitality, their moral and financial support, for being very patient with me during my writing up and for their advice and encouragement. I would also like to thank them for being very thoughtful and friendly, for their major contribition to the improvement of my spoken as well as written English, and for being always helpful when ever I had a problem. I am also very grateful to all their friends and relatives; my special thanks are due to Mrs Anna Maria Fubini, Mrs Frankie Goring, Mr Stuart and Mrs Marian Goring, Mrs Teddy Gross, Drs Micheal and Margeret Hathorn, Drs Geoffrey and Trudi Holmes, Mr Hannes and Mrs Alice Keller, Mr Ernest Mitchell, Dr Martin Rosendaal, Dr Otto and Mrs Helena Safir, Dr Stephen Wight, Mr Michael and Mrs Betty Weinstein for their help, advice and for being very friendly and thoughtful.

I would like to thank my father and mother for their support during my education and for encouraging me to persue my studies in spite of all the social conflicts. My special thanks are due to my brother Lounés, without his help and influence, I would not have had a chance to continue my studies.

LIST OF CONTENTS

	Abstract	3
	Acknowledgments	5
	List of figures	15
	List of Tables	19
	List of abreviations	21
	CHAPTER 1. GENERAL INTRODUCTION	26
1.1.	Embryology and structure of the testis	27
1.1.1.	Structure of the seminiferous tubules	28
1.1.2.	Structure of the testicular interstitium	29
1.1.2.1.	Leydig cells	29
1.1.2.2.	Macrophages	31
1.2.	Leydig cell morphology	
1.2.1.	Leydig cell size	32
1.2.2.	Leydig cell ultrastructure	32
1.2.2.1.	The smooth endoplasmic reticulum	33
1.2.2.2.	The lipid droplets	33
1.2.2.3.	The nucleus	34
1.2.2.4.	The mitochondria	34
1.2.2.5.	The peroxisomes	35
1.2.2.6.	The cytoskeleton	35
1.2.2.7.	The Golgi complexes	36
1.2.3.	The mode of secretion of testosterone	36
1.3.	Leydig cell steroidogenesis	37
1.3.1.	Sources of cholesterol	38
1.3.1.1.	Uptake of plasma lipoprotein particles	39
1.3.1.2.	*De novo* synthesis of cholesterol	39
1.3.1.3.	Intracellular storage of cholesterol	41
1.3.1.4.	Plasma membrane storage of cholesterol	42
1.3.2.	Mobilization of cholesterol	43
1.3.2.1.	Role of *de novo* protein synthesis	44
1.3.2.1.1.	Sterol carrier protein-2 (SCP_2)	45
1.3.2.1.2.	Steroidogenesis activator polypeptide (SAP)	45
1.3.2.1.3.	"8.2K" protein	45

1.3.3.	Conversion of cholesterol to pregnenolone by $P450_{scc}$	47
1.3.3.1.	Components of the enzyme system and their interactions	47
1.3.3.2.	Metabolism of hydroxysterols	49
1.3.3.3.	Regulation of $P\text{-}450_{scc}$	51
1.3.4.	Metabolism of pregnenolone	53
1.3.4.1.	3ß-Hydroxysteroid dehydrogenase-isomerase (3ß-HSD)	54
1.3.4.2.	17α-Hydroxylase and C_{17-20} lyase ($P\text{-}450_{17\alpha}$)	55
1.3.4.3.	17-Ketosteroid reductase and 17ß-hydroxysteroid dehydrogenase	58
1.3.4.4.	5α-Reductase	59
1.3.4.5.	Aromatase	60
1.3.4.5.1.	Structure and cellular localization of aromatase	60
1.3.4.5.2.	Mechanism of aromatization	61
1.3.4.5.3.	Tissue distribution of aromatase	63
1.3.4.5.4.	Regulation of aromatase activity and/or synthesis	64
1.3.4.6.	Oestradiol-17ß hydroxysteroid dehydrogenase	69
1.4.	Regulation of Leydig cell function	70
1.4.1.	The hypothalamic and pituitary control	70
1.4.1.1.	structure of luteinizing hormone and human chorionic gonadotrophin	70
1.4.1.2.	The role of carbohydrate moities in LH/hCG function	71
1.4.1.3.	The hypothalamic control of pituitary gonadotrophins	72
1.4.1.4.	The control of pituitary gonadotrophins by gonadal peptides	76
1.4.1.5.	The control of pituitary gonadotrophins by gonadal steroids	77
1.4.2.	The adrenal control (glucocorticoids)	79
1.4.3.	Intratesticular control	82
1.4.3.1.	Leydig cell-peritubular cell interactions	83
1.4.3.2.	Leydig cell-macrophage interactions	84
1.4.3.3.	Leydig cell-Sertoli cell interactions	84
1.4.3.4.	Peritubular cell-Sertoli cell interactions	90
1.4.3.5.	Sertoli cell-germinal cell interactions	91
1.4.3.6.	Lymphocyte-Leydig and Sertoli cell interactions	92
1.4.3.7.	Mast cell-Leydig cell interactions	93
1.4.3.8.	Autocrine regulation of Leydig cell function by corticotrophin releasing hormone (CRF)	93
1.5.	The mechanisms of action of luteinizing hormone	94
1.5.1.	Luteinizing hormone receptor	94
1.5.1.1.	Structure of LH receptor	95

1.5.2.	LH receptor transducing systems	98
1.5.2.1.	Transducing systems involving cyclic AMP	100
1.5.2.1.1.	GTP Binding proteins	100
1.5.2.1.2.	Adenylate cyclase and cyclic AMP-dependent protein kinase	101
1.5.2.2.	Transducing system involving inositol phospholipid metabolism	104
1.5.2.2.1.	The phosphoinositide cycle	105
1.5.2.2.2.	The role of Ca^{2+}	106
1.5.2.2.3.	The role of protein kinase C and diacyl glycerol	107
1.5.2.2.4.	Role of arachidonic acid and its metabolites	109
1.5.3.	Regulation of LH receptor	111
1.5.3.1.	Desensitization and down-regulation	111
1.5.3.1.1.	Mechanisms involved in the down-regulation of LH/hCG receptor	113
1.5.3.1.2.	Mechanisms involved in the desensitization of LH receptor	115
1.5.3.2.	Inhibition of steroidogenesis in LH/hCG-desensitized cells: role of testicular oestrogens	116
1.6.	Leydig cell development	119
1.6.1.	Leydig cell precursors	120
1.6.2.	Regulation of Leydig cell development	121
1.6.2.1.	Regulation of Leydig cell growth by LH/hCG	122
1.6.2.2.	regulation of Leydig cell growth by testicular factors	124
1.6.2.3.	The possible mechanisms involved in Leydig cell growth induced by LH/hCG and testicular factors	126
1.7.	Development of Leydig cell tumours	130
1.7.1.	Occurence	130
1.7.2.	Efffects of cryptorchidism	133
1.7.3.	Morphological changes which precede the development of Leydig cell tumours	133
1.7.4.	Changes in hormone levels which precede the development of Leydig Cell tumours	135
1.7.5.	Induction of Leydig cell tumours	137
1.7.5.1.	Induction of Leydig cell tumours in the mouse	137
1.7.5.2.	Induction of Leydig cell tumours in the rat	139
1.7.5.2.1.	The role of oestrogens in the induction of Leydig cell tumours by LH/hCG	140
1.7.6.	The mechanism(s) of tumour development	142
1.7.6.1.	Involvement of paracrine factors	142

1.7.6.2.	The role of peroxisomes in tumour development	144
1.7.6.3.	The possible involvement of inhibin in tumour development	145
1.7.6.4.	The role of macrophages in tumour development	145
1.8.	Effect of dopamine and its agonists on the hypothalamic-pituitary-adrenal and gonadal axis	147
1.8.1.	Dopamine receptors	150
1.8.2.	Pharmacology and clinical application of mesulergine	153
1.8.3.	Effects of dopamine and its agonists on anterior pituitary hormones	153
1.8.3.1.	Effects of dopamine and its agonists on prolactin synthesis and release	157
1.8.3.2.	Effects of dopamine and its agonists on LH secretion	160
1.8.3.3.	Effects of dopamine and its agonists on growth hormone secretion	160
1.8.4.	Effects of ageing on the hypothalamic pituitary-adrenocortical and gonadal axis: alteration in the responsiveness to dopamine and its agonists	160
1.8.5.	Effects of PRL on Leydig cell function	161
1.8.6.	Direct effect of dopamine and its agonists on the testis	162
1.8.7.	Effects of dopamine and its agonists on Leydig cell proliferation	163
1.9.	AIMS	164
	CHAPTER 2. METHODS	165
2.1.	Materials	166
2.2.	Treatment of animals	168
2.2.1.	Dopamine agonist experiments	168
2.2.2.	hCG and dexamethasone experiments	169
2.3.	Collection of testicular interstitial fluid	169
2.4.	Interstitial cell preparation	170
2.4.1.	Collagenase digestion	170
2.4.2.	Elutriation and Percoll purification	171
2.4.3.	Isolation of macrophages using Dynabeads (M-450) coated with ED2	173

2.5.	Determination of Leydig cell volume	175
2.6.	3ß-HSD and diaphorase cytochemistry	175
2.7.	Macrophage Immunocytochemistry	177
2.8.	Leydig cell incubations	178
2.9.	Binding of ^{125}I-hCG to Leydig cells	178
2.10.	Testosterone, pregnenolone and cyclic AMP assays	179
2.11.	Prostaglandin $F_{2\alpha}$ assay	181
2.12.	Aromatase assay	181
2.13.	Protein synthesis	182
2.14.	Histological and immunohistological procedures	183
2.15.	Cell counts	184
2.16.	Analysis of data	185
	RESULTS	
	CHAPTER 3. SEPARATION AND CHARACTERIZATION OF LEYDIG CELLS AND MACROPHAGES FROM RAT TESTES	186
3.1.	Summary	187
3.2.	Introduction	189
3.3.	Results	190
3.3.1.	Leydig cell content	190
3.3.2.	Macrophage content	191
3.3.3.	Leydig cell volume	191
3.3.4.	Specific binding of ^{125}I-labelled hCG to Leydig cells and testosterone and cAMP production	196
3.4.	Discussion	202

	CHAPTER 4. EFFECTS OF A DOPAMINE AGONIST MESULERGINE (CU 32-085) ON LEYDIG CELL FUNCTION	206
4.1.	Summary	207
4.2.	Introduction	209
4.3.	Results	210
4.3.1.	Food intake, body weight, testicular and seminal vesicle size, and interstitial fluid volume	210
4.3.2.	Effect of *in vivo* treatment with CU 32-085 on LH receptors and steroidogenesis in rat Leydig cells	210
4.3.2.1.	Specific binding of ^{125}I-labelled hCG to Leydig cells	210
4.3.2.2.	Testosterone and Cyclic AMP production	214
4.3.2.3.	The metabolism of 22R-hydroxycholesterol	216
4.3.2.4.	Aromatase activity	218
4.3.3.	Effect of *in vivo* treatment with CU 32-085 on LH receptors and steroidogenesis in elutriated/Percoll-purifed Leydig cells separated according to their different sedimentation velocities	220
4.3.4.	Effect of *in vitro* treatment with CU 32-085 on steroidogenesis in elutriated/Percoll-purified Leydig cells	225
4.4.	Discussion	227
	CHAPTER 5. EFFECTS OF A DOPAMINE AGONIST CU 32-085 ON LEYDIG CELL PROLIFERATION	233
5.1.	Summary	234
5.2.	Introduction	236
5.3.	Results	239

5.3.1. Effect of treatment *in vivo* with the DA for 5 weeks on protein synthesis in elutriated/Percoll-purified Leydig cells 239

5.3.2. Effect of treatment *in vivo* with the DA for 1,2,4,5, or 12 weeks on the number of Leydig cells and macrophages in crude testicular interstitial cells 239

5.3.3. Effect of treatment *in vivo* with the DA for 5 or 12 weeks on the distribution of Leydig cells according to their sedimentation velocities 242

5.3.4. Histological analysis of Leydig cell tumour development and determination of the number of Leydig cells and macrophages in testicular sections from control rats and rats treated *in vivo* with the DA for 5 or 57 weeks 244

5.3.5. Inhibin-like immunoreactivity in testicular sections from control rats and rats treated *in vivo* with the DA for 57 weeks 251

5.4. Discussion 254

CHAPTER 6. EFFECTS OF hCG AND DEXAMETHASONE ON LEYDIG CELL FUNCTIONS 263

6.1. Summary 264

6.2. Introduction 266

6.3. Results 268

6.3.1. Effect of treatment *in vivo* with hCG and/or dexamethasone for 1 week on body weight, and testicular and seminal vesicle weight 268

6.3.2. Effect of treatment *in vivo* with hCG/dexamethasone for 6h or 1week on LH receptors and steroidogenesis in elutriated/Percoll-purified Leydig cells 269

6.3.2.1. Specific binding of ^{125}I-labelled hCG to Leydig cells 269

6.3.2.2. Testosterone and cAMP production 269

6.3.2.3. Aromatase activity 273

6.3.3. Effect of treatment *in vivo* with hCG and/or dexamethasone for 6h on $PGF_{2\alpha}$ production 273

6.3.4. Effect of addition of dexamethasone *in vitro* on the responsiveness of the Leydig cells isolated from control rats and rats treated with the dopamine agonist (CU 32-085) 276

6.4. Discussion 278

CHAPTER 7. GENERAL DISCUSSION 282

7.1. The possible mechanisms involved in the effect of the DA on Leydig cell function(s) in rats 283

7.2. The possible mechanisms involved in the development of Leydig cell tumours as a result of treatment with the DA in rats 288

7.3. Safety assessment of CU 32-085 in man: is the extrapolation of the finding in the rat valid 291

7.4. Development of a method for the separation of Leydig cells from macrophages and the study of Leydig cell heterogeneity 294

7.5. Proposals for further work 295

APPENDIX 1: Methods 298

APPENDIX 2: Chapter 4 and 5 321

APPENDIX 3: Chapter 6 328

REFERENCES 333

PUBLICATIONS AND COMMUNICATIONS 392

LIST OF FIGURES

1.1.	Cross-section through part of an adult testis	30
1.2.	Possible intermediates in the formation of pregnenolone from cholesterol	50
1.3.	Pathways involved in the biosynthesis of testicular steroids from cholesterol	56
1.4.	Aromatase reaction sequence	62
1.5.	The chemical structure of two aromatase inhibitors; aminoglutethimide and 4-hydroxyandrostenedione (4OHA)	68
1.6.	Diagrammatic summary of the regulation of FSH and LH secretion in the male	75
1.7.	Postulated topology of the LH receptor in the plasma membrane	97
1.8.	Transducing systems involved in the action of LH/hCG on Leydig cells	99
1.9.	Pathways for the generation and metabolism of arachidonic acid	112
1.10.	Model of signal transduction at the D2 receptor	148
1.11.	Chemical structure of dopamine, mesulergine (CU 32-085) and its 1, 20-N, N-bidemethylated metabolite of of mesulergine	151
1.12.	Schematic diagram showing some of the postulated neurochemical interactions which may control GnRH secretion	158
3.1.	Macrophage and Leydig cell content in elutriated and elutriated/Percoll-purified interstitial cell fractions from rat testes	193
3.2.	Leydig cell volume in elutriated/ Percoll purified testicular interstitial cell fractions	195

3.3. Specific binding of ^{125}I-labelled hCG to Leydig cells and production of testosterone and cAMP in elutriated/ Percoll-purified interstitial cell fractions from rat testes 198

3.4. Basal and LH-stimulated testosterone and cAMP production by elutriated/Percoll-purified Leydig cells (El.+ Percoll) and Leydig cells further purified with Dynabeads (M-450) coated with a macrophage monoclonal antibody ED2 (EL. + Percoll + Dyn.) 199

4.1. Specific binding of ^{125}I-labelled hCG to Leydig cells in crude and elutriated/Percoll-purified testicular interstitial cells from testes of control and DA-treated rats 212

4.2. Scatchard analysis of the binding of ^{125}I-labelled hCG to elutriated/Percoll-purified Leydig cells from testes of controls and rats treated with the DA for 5 weeks. 213

4.3. Testosterone and cAMP production in elutriated/Percoll-purified Leydig cells from control rats and rats treated with a DA for 5 weeks 215

4.4. Testosterone and pregnenolone production in elutriated/ Percoll-purified Leydig cells from testes of control rats and rats treated with the DA for 5 weeks 217

4.5. Aromatase activity in elutriated/Percoll-purified testicular interstitial cells from testes of control rats and rats treated with the DA for 4 weeks. 219

4.6. Specific binding of ^{125}I-labelled hCG to Leydig cells and production of testosterone and cAMP in elutriated/Percoll-purified interstitial cells, from testes of control rats and rats treated with the DA for 5 weeks, separated according to their different sedimentation velocities 222

4.7. Macrophage and Leydig cell content in elutriated/Percoll-Purified interstitial cell fractions from testes of control rats and rats treated with the DA for 5 weeks 223

4.8. *In vitro* effects of the DA on testosterone and cAMP production in elutriated/Percoll-purified Leydig cells from rat testes 226

5.1 Protein synthesis in elutriated/Percoll purified testicular interstitial cells in control and DA-treated rats 240

5.2. Number of Leydig cells and macrophages in crude testicular interstitial cells from controls and DA-treated rats 241

5.3. Leydig cell distribution according to their sedimentation velocities in control and DA-treated rats 243

5.4. Number of Leydig cells and macrophages present in the interstitial tissue of testes from control and DA-treated rats 245

5.5. Representative cross-sections of testes from 13-week-old control rats and rats treated with the DA for 5 weeks 248

5.6. Representative cross-sections of testes from 65 week-old control rats and rats treated with the DA for 57 weeks 249/50

5.7. Inhibin-like immunoreactivity in representative cross sections of testes from controls and rats treated with the DA for 57 weeks 252/53

6.1. Specific binding of ^{125}I-labelled hCG to Leydig cells from testes of control rats and rats treated with hCG and/or dexamethasone 271

6.2. Testosterone and cAMP production in elutriated/Percoll-purified Leydig cells from testes of control rats and rats treated with hCG and/or dexamethasone 272

6.3. Aromatase activity in elutriated/Percoll-purified testicular interstitial cells from testes of control rats and rats treated with hCG and/or dexamethasone 274

6.4. The effects of hCG and dexamethasone *in vivo* on $PGF_{2\alpha}$ production by Leydig cells *in vitro* 275

6.5. Effect of dexamethasone *in vitro* on the responsiveness of the Leydig cells isolated from control rats and rats treated with dopamine agonist *in vivo* 277

7.1. The possible mechanisms involved in the effect of the dopamine agonist (CU 32-085) on Leydig cell functions and growth in Sprague Dawley rats 293

A1.1. Typical standard Percoll density gradient profile 300

A1.3. Typical elution profile of free ^{125}I and ^{125}I-labelled hCG from a PD-10 Sephadex G-25M column (a) and a competitive binding curve for the characterization of ^{125}I-labelled hVG using increasing concentrations of unlabelled hCG (CR 127) 307

A1.4. Typical profile of separation of free ^{125}I and ^{125}I-ScAMP-TME by descending paper chromatography or using Sep-Pak^{R} cartridges 310

A1.5. Typical standard curve for the radioimmunoassay of cAMP 313

A1.6. Typical standard curve for the RIA of pregnenolone and testosterone 316

A2.1. Food intake in controls and rats treated with a dopamine agonist (DA) (CU 32-085) for 4 weeks 322

A2.2. Body weight in control and DA-treated rats 323

A2.3. Testicular and seminal vesicle size in control and DA-treated rats 324

A2.4. Testicular weight and interstitial fluid volume in control rats and rats treated with the DA for 5 weeks 325

A3.1. Testicular and seminal vesicle weight in control rats and rats treated with hCG and/or dexamethasone 330

LIST OF TABLES

1.1.	Distribution of testicular tumours according to histological type	131
2.1.	Sedimentation velocities of elutriated testicular interstitial cells	172
3.1	Leydig cell and macrophage content in crude testicular interstitial cells	192
3.2.	Macrophage content in the fraction corresponding to fractions F4 to F8 after elutriation, purification on Percoll gradients, and a further purification with Dynabeads coated with a macrophage monoclonal antibody	194
3.3.	Cell viability in elutriated/Percoll purified testicular interstitial cell fractions and in the cell fraction further purified with Dynabeads (M-450) coated with a macrophage monoclonal antibody	200
3.4.	Effect of macrophage enriched testicular interstitial cells on testosterone production by elutriated/Percoll purified Leydig cells	201
4.1.	Viability of elutriated/Percoll-purified Leydig cells, from testes of control rats and rats treated with the DA for 5 weeks, separated according to their different sedimentation velocities	224
5.1.	Incidence of Leydig cell tumours in rats given the dopamine agonist (CU 32-085) for 2½ years	237
5.2.	Mean nuclear diameter of Leydig cells from control rats and rats treated with the DA for 5 or 57 weeks	246
A1.1.	Leydig cell densities after elutriation/Percoll-purification (band 2)	301
A1.2.1.	Determination of Leydig cell content in unpurified interstitial cell preparation by 3ß-HSD cytochemistry and phase contrast microscopy	303

A1.2.3. Leydig cell and macrophage content in crude testicular interstitial cells purified on Percoll 305

A1.7. Determination of a possible loss in ^3H_2O resulting from aromatization by evaporation during incubation at 34°C for 2h 317

A2.1. Viability of elutriated/Percoll-purified Leydig cells, from testes of control rats and rats treated with the DA for 5 weeks 326

A2.2. Incidence of death in control rats and rats treated with the dopamine aginist for 71 weeks 327

A3.1. Body weight in control rats and rats treated with hCG and/or dexamethasone for one week 329

A3.2. Viability of elutriated/Percoll-purified Leydig cells from testes of control rats and rats treated with hCG and/or dexamethasone 331

A3.3. Macrophage content in crude testicular interstitial cells and in elutriated/Percoll-purified Leydig cell preparations 332

LIST OF ABBREVIATIONS

AII	angiotensin-II
A23187	calcium ionophore
AA	arachidonic acid
ABP	androgen binding protein
AC	adenylate cyclase
ACTH	adrenocorticotrophic hormone
ACAT	cholesterol acyltransferase
AP-2E	activating protein-2-binding elements
AVP	arginine vasopressin
AY 27-110	dopamine agonist (nonergoline)
BBS	bonbesin
bFGF	basic FGF
3ßHSD	3ß-hydroxysteroid dehydrogenase
11ßHSD	11ß-hydroxysteroid dehydrogenase
8brcAMP	8-bromo-cAMP
BSA	bivine serum albumin (fraction V)
C	catalytic subunit of PK-A
cAMP	adenosine 3':5'-cyclic monophosphate
cDNA	complementary DNA
CEH	cholesteryl ester hydrolase
c-fos	proto-oncogene
CF 25-397	dopamine agonist (ergoline)
CH 29-717	N1 demethyllated metabolite of mesulergine
c-myc	proto-oncogene
CR127	batch number of hCG
CRF	corticotrophin releasing factor
CTX	cholera toxin
CU 32-085	1,6-dimethyl-8α-N.N-dimethyl sulfanoyl amino-ergoline (mesulergine)
CV 205-502	dopamine agonist
Cyanoketone	inhibitor of 3ß-HSD
D1 receptor	dopamine D1 receptor
D2 receotor	dopamine D2 receptor
D3 receptor	dopamine D3 receptor
DA	dopamine agonist (mesulergine, CU 32-085)
DAB	diaminobenzidine-tetrachloride
DAG	diacylglycerol
DBI	diazepam binding inhibitor
dcAMP	dibutyryl cAMP
DePex	non-aqueous mounting medium
Dex	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMEM/F12	DMEM and Ham's nutrient mixture F-12 base
DMF	dimethylformamide

DNA	deoxyribonucleic acid
dpm	desintegration per min
Dyn	purified with Dynabeads
Dynabeads M-450	magnetic beads coated with sheep anti-mouse IgG
E2	oestradiol-17ß
E2-17ßHSD	E2-hydroxysteroid dehydrogenase
EBSS	Earles balanced salt solution
ED2	rat macrophage monoclonal antibody
EDS	ethane-1,2-dimethane sulphonate
EDTA	ethylene-diaminetetra-acetic acid
EGF	epidermal growth factor
El	elutriated
EOPs	endogenous opioids
ER	endoplasmic reticulum
ETOH	ethanol
F	fraction
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
GABA	gamma-aminobutyric acid
GAP	gonadotrophin-releasing hormone associated peptide
GH	growth hormone
G_i	G-protein which mediates inhibition of adenylate cyclase
G_k	G-protein which mediates stimulation of K^+ channels
GnRH	gonadotrophin releasing hormone
G_p	G-protein which mediates stimulation of PL-C and PLA_2
G_s	G-protein which mediates stimulation of adenylate cyclase
H_2 receptors	histamine receptors
H_2O_2	hydrogen peroxide
H-540 cells	tranplantable rat tumour Leydig cells
HETEs	hydroxyeicosatetraenoic acids
hCG	human chorionic gonadotrophin
$HClO_4$	perchloric acid
HDL	high-density lipoprotein
HDN	hypothalamic dopaminergic neurons
Hepes	N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPA	hypothalamic-pituitary-adrenal axis
HPG	hypothalamic-pituitary-gonadal axis
IBMX	3-isobutyl-1-methylxanthine
IF	interstitial fluid
IGF	Insulin-like growth factor
IgG	immunoglobilin G

IL	interleukin
INF-gamma	interferon-gamma
i.p.	intraperitoneal
IP_3	inositol trisphosphate
KD	dissociation constant
17-KSR	17-ketosteroid reductase
LDL	low-density lipoprotein
LH	ovine luteinizing hormone (oLH)
LHRH	luteinizing hormone releasing hormone
LTB_4	leukotrine B_4
MA10 cells	mouse tumour Leydig cells
MCF-7 cells	oestrogen receptor positive breast cancer cells
MDA-MB cells	oestrogen receptor negative cells
MPC	magnetic particle concentrator
mPPAR	mouse peroxisome proliferator activated receptor
mRNA	messenger RNA
MS1	cAMP antibody
α-MSH	α-melanocyte-stimulating hormone
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	sodium hydroxide
NaN_3	sodium azide
NGF	nerve growth factor
NSB	non-specific binding
NTS	neurotensin
OAC	ovine adrenocortical cells
ODN	octadecaneuropeptide
4OHA	4-hydroxyandrostenedione
P450	cytochrome P-450
$P\text{-}450_{AROM}$	P-450-dependent aromatase
$P\text{-}450_{17\alpha}$	P-450-depenednt 17α-hydroxylase, $C_{17\text{-}20}$ lyase enzyme
$P\text{-}450_{scc}$	P-450-dependent side chain cleavage complex
PAS	periodic acid Schiff
PBDR	peripheral benzodiazepine receptors
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PEG	polyethylene glycol 6000
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGs	prostaglandins
PGI_2	prostacyclin
PI	phospatidylinositol
PIP_2	PI bis-4,5-phosphate
PKA	protein kinase-A
PKC	protein kinase-C

PKCI	PK-Cgamma
PLA_2	phospholipase-A_2
PLC	phospholipase-C
PLD	phospholipase-D
PMA	phorbol-12-myristate-13-acetate
PModS	peritubular factor which modulate Sertoli cell function
p.o.	oral
POMC	pro-opiomelanocortin
PRL	prolactin
PTX	pertussis toxin
R	regulatory subunit of PK-A
22R	22R-hydroxycholesterol
R2C	rat tumour Leydig cells
RIA	radioimmunoassay
RNA	ribonucleic acid
R_p-cAMP	cAMP analogue
RT	room temperature
SAP	steroidogenesis activator polypeptide
SCP_2	Sterol carrier protein 2
SER	smooth endoplasmic reticulum
s.c.	sub-cutaneous
ScAMP-TME	succinyl cAMP tyrosine methyl ester
SCSGF	Sertoli cell-secreted growth factor
SCSP	Sertoli cell-secreted proteins
SD	standard deviation
SDZ 200-110	calcium channel blocker
SGF	seminiferous tubule growth factor
SLCT	Sertoli-Leydig cell-tumour
SU-10603-10603	7-chloro-3, 4-dihydro-2-(3-pyridyl)-1-(2H)-naphthalenone (inhibitor of 17α-hydroxylase)
T_3	throid hormone
TCA	trichloroacetic acid
TGF	transforming growth factor
TNF	tumour necrosis factor
TPA	12-O-tetradecanoylphorbol 13-acetate
TRH	thyrotrophin releasing hormone
Tris/HCl	Tris(hydroxymethyl)aminomethane hydrochloride (TRIZMA^R hydrochloride)
Tris/base	TRIZMA^R base
TSH	thyroid-stimulating hormone
Tx	thromboxane
VIP	vasoactive interstinal peptide
v/v	volume/ volume
w/v	weight/ volume

UNITS AND MEASURES

Centrifugal force	g (gravitational force)
Concentrations:	IU (international units), mol (moles), and M (molar = mol/l)
Density	(g/ml)
Temperature:	°C (degrees celsius)
Time:	h (hours), min (minutes), and sec (seconds)
Mass:	Da (Daltons) and g (grams)
Radioactivity	Bq (becquerel = 1 disintegration per second) Ci (curie = 3.7×10^{10} Bq = 2.22×10^{12} cpm) cpm (counts per minute)

Sedimentation Velocity

S $1.93 \times f/R^2$ (millimeter per hour/g = mm/h·g)

g	centrifugal force
f	volumetric flow rate (ml/min)
R	rotor speed expressed in units of 10^3rpm
rpm	revolutions per min
1.93	constant factor involving chamber and rotor characteristics and conversion from minutes to hours and centimeters to millimeters

Standard prefixes

Prefix	Symbol	Factor
giga	G	$\times 10^9$
mega	M	$\times 10^6$
kilo	k	$\times 10^3$
milli	m	$\times 10^{-3}$
micro	μ	$\times 10^{-6}$
nano	n	$\times 10^{-9}$
pico	p	$\times 10^{-12}$
femto	f	$\times 10^{-15}$

Volume: l litres

CHAPTER 1

GENERAL INTRODUCTION

The testis is a compound gland which includes seminiferous tubules and interstitial tissue. These two compartments are closely interlinked, thereby enabling the testis to fulfill its dual function of spermatogenesis and steroidogenesis. The size of the testis varies among species, it occupies about 1% of the total body weight in rodents (Setchell, 1978) while in man and some monkeys, this percentage is smaller (Harcourt *et al.*, 1981).

1.1. EMBRYOLOGY AND STRUCTURE OF THE TESTIS

The testis derives from the gonadal ridge which gives rise to the Sertoli cells of the tubules and also to the interstitial cells of the intertubular tissue. The germ cells are derived from the endodermal lining of the yolk sac. The foetal testis plays a dominant role in the development of the male internal and external genitalia although, the spermatogenic function of the testis does not begin until puberty. The regression of the Mullerian ducts in the male foetus is triggered by secretion of the Mullerian-inhibiting factor (MIF) by Sertoli cells. Differentiation of the Wolffian ducts into the epididymis, the vas, and the seminal vesicles is dependent upon testosterone produced by the foetal Leydig cells.

The mammalian testis is composed of the avascular seminiferous tubules and vascularized interstitial tissue. In most mammals, the seminiferous tubules account for over 90% of the volume of the testis, although in some species such as the boar, 40% or more of the volume may be taken up by interstitial tissue (Fawcett *et al.*, 1973). In the rat testis, 82.4% of the volume is occupied by seminiferous tubules, 15.7% by the interstitial tissue, and 1.9%

by the capsule (Mori & Christensen, 1980).

1.1.1. Structure of the seminiferous tubules

The seminiferous tubules contain germ cells at different stages of spermatogenesis and the non-proliferating somatic cells, the Sertoli cells (Figure 1.1). The main function of the seminiferous tubules is spermatogenesis which is a complex process involving the development of stem cells into spermatozoa, via the intermediate formation of spermatogonia, spermatocytes and spermatids. The developing germ cells are enfolded by the somatic component of the seminiferous epithelium, the Sertoli cells. In addition, adjacent Sertoli cells are connected through tight junctions thus forming a Sertoli cell or blood-testis barrier. This barrier divides the tubules into basal and adluminal compartments. Selective passage of compounds through this barrier combined with active secretion of compounds produced by Sertoli cells, probably results in an optimal environment for developing germ cells. Germ cells develop in a cyclic manner during migration from the basal to the adluminal site of the seminiferous epithelium. These cellular organizations in the rat were classified into 14 stages of the seminiferous epithelial cycle by Leblond & Clermond (1952). Sertoli cells are known to undergo morphological (Ueno & Mori, 1990) and functional (Morales *et al.*, 1987) changes during the spermatogenic cycle. The seminiferous epithelium is surrounded by a tubular wall composed of four layers. In rodents, these are identified as an innermost layer of non-cellular material which is surrounded by a layer of smooth muscle-like or myoid cells, a layer of collagen fibers, and on outside a layer

of endothelial cells that line the lymphatic space in the interstitial tissue (Setchell, 1978).

1.1.2. Structure of the testicular interstitium

Interstitial tissues of mammalian testes consist of a variety of cell types including Leydig cells, macrophages, endothelial cells, fibroblasts, components of the blood and lymphatic system, and a variable amount of connective tissue (de Kretzer & Kerr, 1988; Pöllänen & Maddocks, 1988) (Figure 1.1). In many mammals including laboratory rodents, man, rhesus monkeys and the ram, lymphatic vessels are a prominent feature of the interstitial area. In rodents such as the rat, mouse, and guinea-pig these lymphatics are particularly extensive (Fawcett *et al.*, 1973). In all of the species with prominent lymphatics, there is abundant interstitial fluid bathing the Leydig cells, blood vessels and the outside of the seminiferous tubules. It is via this fluid that all hormones and nutrients are transported from the blood stream to the testicular cells and between the cells within the testis (Fawcett *et al.*, 1973).

1.1.2.1. Leydig cells

Leydig cells are the most conspicuous cell type in interstitial tissues of many species. In the rat testis, Leydig cells constitute 2.7% of testicular volume, each cubic centimeter of the testis contains about 22 million Leydig cells (Mori & Christensen, 1980). Tight junctions were found between closely adjacent cells (Ohata, 1979). In rats it has been found that the Leydig cells lying near the tubules undergo changes in size and structure depending on the stage of spermatogenesis in the adjacent tubules (Bergh, 1982, 1983).

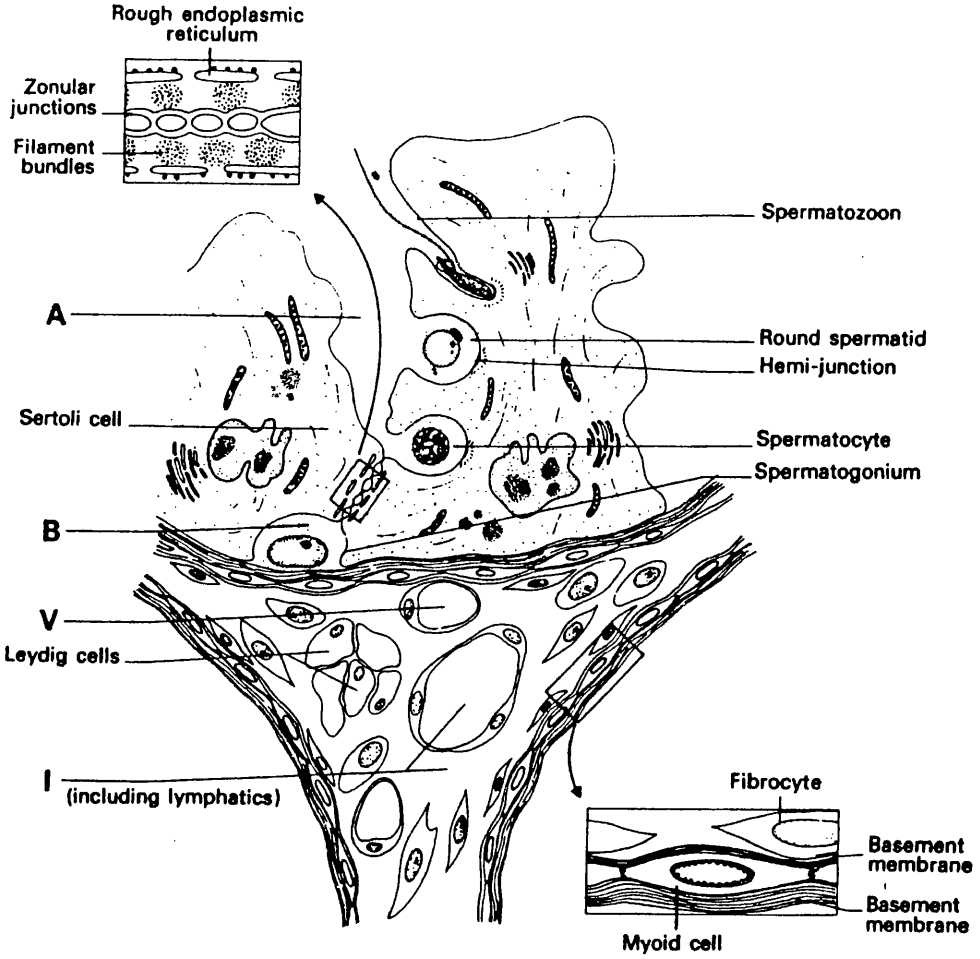


Figure 1.1. Cross-section through part of an adult testis.
There are four compartments in the testis: V= vascular; I= interstitial including the lymphatic vessels and containing the Leydig cells, macrophages, and endothelial cells; the basal (B) and adluminal (A) compartments lie within the seminiferous tubules. Within the basal compartment are the spermatogonia, whilst spermatocytes, round spermatids and spermatozoa are in the adluminal compartment, and in intimate contact with the Sertoli cells which form anchoring 'hemi-junctions' with the elongating spermatids (Johnson & Everitt, 1984).

1.1.2.3. Macrophages

The ovary (Bulmer, 1964; Kirsch *et al.*, 1981; Bagavandoss *et al.*, 1990; Loukides *et al.* 1990), the placenta (Bulmer & Johnson, 1984), and the testis contain macrophages which in the testis reside in the interstitial compartment. These cells are considered to be authentic macrophages because they secrete lysozyme and superoxide anion, have Fc receptors and Ia antigen, are capable of phagocytosis, have high nonspecific esterase activity, and can kill pathogenic organisms (Yee & Hutson, 1983; Reiko & Werb, 1984; Johnston, 1988; Hutson, 1989). Macrophages are a prominent cellular component of testicular interstitial tissues of several species including mice (Ohata, 1979), ram (Pöllänen & Maddocks, 1988), man (El-Demiry *et al.*, 1987), and rats (Miller *et al.*, 1983; Bergh, 1985, 1987; Pöllänen & Maddocks, 1988, Hutson, 1990; Raburn *et al.*, 1991). In rats, macrophages are first identifiable within the testis at day 19 of foetal development and subsequently increase 4-6 fold in concentration within the interstitium between day 10 and 20 postpartum and an additional increase of 15 fold between day 21 and 56 post partum (Hardy *et al.*, 1989; Hutson, 1990). The increase in the number of macrophages was suggested to be under the control of LH, since treatment *in vivo* with hCG was found to result in an increase in the number of macrophages in neonatal rat testis (Raburn *et al.*, 1991). There is also a 2 fold increase in the size of adult macrophages compared with those measured in prepubertal animals (Hutson, 1990). Macrophages commonly reside within the clusters of Leydig cells, where they form specialized junctional complexes (Miller *et al.*, 1983).

1.2. LEYDIG CELL MORPHOLOGY

It has been known for many years that the testis plays a major role in the control of male characteristics. The interstitial cells of the testis were first described by Leydig (1850). The early evidence for the endocrine function of Leydig cells came from studies by Bouin & Ancel (1903; 1905) and the major hormone secreted by the Leydig cells was identified to be testosterone (Gallagher & Koch, 1929). The control of Leydig cell function by pituitary hormones was first demonstrated by Smith (1930). Hall *et al.* (1969) and subsequently Cooke *et al.* (1972) have shown that conversion of cholesterol to androgens took place in the Leydig cells. This was confirmed by histochemical staining for 3ß-hydroxysteroid dehydrogenase isomerase (3ß-HSD) (Levy *et al.*, 1959; Goldberg *et al.*, 1964; Paz *et al.*, 1980; Molenaar *et al.*, 1984), the determination of 3ß-HSD activity in isolated rat Leydig cells (Paz *et al.* 1982) and homogenates from interstitial cells and seminiferous tubules (van der Vusse *et al.*, 1974), and by immunocytochemistry and *in situ* hybridization (Dupont *et al.*, 1990).

1.2.1. Leydig cell size

There is a diversity in the internal composition and the size of Leydig cells in mammals (de Kretzer & Kerr, 1988). For example rat Leydig cells are polyhedral or sprindle shaped and relatively small, averaging about 10 to 15 µm in diameter (Christensen & Fawcett, 1966), while human Leydig cells are about 15µm in diameter (Christensen, 1975). Leydig cell has a volume of 1,210 μm^3 (Mori & Christensen, 1980).

1.2.2. Leydig cell ultrastructure

Careful morphometric analysis of rat (Mori & Christensen, 1980), mouse (Ohata, 1979), and human (Mori *et al.*, 1982) have provided quantitative information about Leydig cell organelles that can be correlated to their physiological function.

1.2.2.1. The smooth endoplasmic reticulum

The most prominent cytoplasmic organelle is the smooth endoplasmic reticulum (SER) which is the major site for steroidogenic enzymes (Mori & Christensen, 1980). Species differences in abundance of SER are seen in Leydig cells; it is very abundant in guinea pig and mouse (Christensen & Fawcett, 1966; Christensen, 1975) while it is less abundant in rat and man (Christensen & Gillim, 1969). Rat Leydig cell SER was first quantified by Kerr *et al.* (1979) and was found to occupy 39% of the Leydig cell volume. Mori & Christensen (1980) have found that SER occupies a surface area of 10,500 μm^2/cell, which is 6.9% times that of the plasma membrane and is 60% of the total membrane area of the cell. Christensen & Gillim (1969) suggested that the abundance of the smooth endoplasmic reticulum in a steroid-secreting cell may reflect the extent to which the cell makes its own cholesterol instead of taking it up from the plasma. Leydig cells appear to synthesize much of their own cholesterol used in steroidogenesis (Hall, 1970; Hou *et al.*, 1990) and derive only a small portion from the plasma (Chen *et al.*, 1980; Benahmed *et al.*, 1983). A correlation was found between testosterone secretion and the amount of SER (Ewing *et al.*, 1979; Nussdorfer *et al.*, 1980; Zirkin *et al.*, 1980; Mori

& Christensen, 1980; Wing *et al.*, 1984; 1985). This is in contrast to what occurs in the rat adrenal cortex which contains less abundant SER (Volk & Scarpelli, 1964) and where most of the cholesterol used during steroidogenesis is derived from the plasma (Werbin & Chaikoff, 1961).

1.2.2.2. Lipid droplets

Cholesterol resulting from de novo synthesis or taken up from the plasma may be esterified and accumulated in membrane-bound lipid droplets. These lipid droplets are often found in foetal and postnatal Leydig cell cytoplasm, while in mature or adult animals, their number and size vary depending on species (Christensen, 1975; Christensen & Fawcett, 1966), with the mouse having 25 times more than the rat (Christensen & Gillim, 1969; Nussdorfer *et al.*, 1980; Payne *et al.*, 1985). The diversity in lipid droplets content was suggested by Christensen (1975) to be associated with the activity of the Leydig cells; the higher the cell activity, the more cholesterol contained in the droplets must be consumed for the synthesis of steroid, and subsequently active cells have fewer and smaller lipid droplets, while less active cells contain more droplets. This is supported by studies showing a marked depletion of Lipid droplets in Leydig cells from hCG-treated animals (Aoki & Massa, 1972).

1.2.2.3. The nucleus

Leydig cells have a large nucleus (average volume 134 μm^3), with an evident nucleolus (Nussdorfer *et al.*, 1980). The nucleus of the Leydig cell is often located excentrically, and usually has a round or irregular oval shape and a characteristic thin rim of heterochromatin at the periphery. When the Leydig

cells are located near the wall of blood vessels or seminiferous tubules, their nucleus usually conforms to the more elongated shape of the cell by revealing an elliptical form (de Kretzer & Kerr, 1988).

1.2.2.4. The mitochondria

The mitochondria in steroid secreting cells are ovoid or rod-shaped. In the rat, mitochondria occupy about 14% of the cytoplasmic volume (Nussdorfer et al., 1980). An average rat Leydig cell contains 622 mitochondria, measuring on the average 0.35 μm in diameter and 2.40 μm in length. The mitochondrial inner membrane including cristea, another important site of steroidogenic enzymes, has a surface area of 2,920 μm^2/ cell, which is 1.9 times that of the plasma membrane (Mori & Christensen, 1980). Intimate association of mitochondria with the SER membranes has been reported (Christensen, 1975; Ohata, 1979) suggesting a functional association between these organelles in the course of the production of testosterone from cholesterol.

1.2.2.5. The peroxisomes

The Leydig cell cytoplasm also contains the peroxisome or microbody. Immunocytochemical and biochemical techniques revealed the presence of peroxisomes in Leydig cells of rats, mice and guinea pigs (Hurban *et al.*, 1980; Reddy & Svododa, 1972ab; Slesers & Hopkins, 1972). Peroxisomes are also found in other steroid-secreting cells (Hurban *et al.* 1972). The function of peroxisomes in steroid-secreting cells is not clear. Recent evidence suggests its role in steroidogenesis, since a sterol carrier protein 2 (SCP_2) involved in the transport of cholesterol used for steroidogenesis (Vahouny *et al.*, 1983) was

found in peroxisomes (Keller *et al.*, 1989; Mendis-Handagama *et al.*, 1990). Treatment with LH was found to result in an increase in rat Leydig cell peroxisome volume and intraperoxisomal SCP_2 content (Mendis-Handagama *et al.*, 1990).

1.2.2.6. The cytoskeleton

Cytoskeletal filaments are also found in Leydig cell cytoplasm including actin and vimentin (Russel *et al.*, 1987) which are found to be involved in the transport of cholesterol to the mitochondria in response to LH and dcAMP (Hall *et al.*, 1979; Silavin *et al.*, 1984; Nagy & Freeman, 1990) and also in the process of downregulation of LH receptors (Law *et al.*, 1984).

1.2.2.7. The Golgi Complexes

Golgi complexes are also present in Leydig cells. Though their function is unknown in steroid secreting cells, their different appearances reflect changes in Leydig cell function (Ohata, 1979).

1.2.3. The mode of secretion of testosterone

The surface area of Leydig cells is very large as a result of the presence of many filipodia and microvilli (Christensen, 1975; Ohata, 1979) which may facilitate the secretion of testosterone. Although it is well established that the site of steroid synthesis in Leydig cells is the membranous system of SER, little is known with regard to the mode of steroid secretion. Christensen (1975) proposed three possible mechanisms of the transport of formed testosterone to the cell surface: (1) the steroid may be transported through the cytoplasmic matrix, (2) transported through the cavity of SER, or (3)

transferred from the SER to vacuoles by pinching off of the latter. Testosterone released by the Leydig cells may enter the blood, the testicular lymph, and the seminiferous tubules where it is used during spermatogenesis. A specific saturable carrier-mechanism probably involving facilitated diffusion has been shown to be involved in the transport of testosterone into the seminiferous tubule fluid (Setchell *et al.*, 1978).

1.3. LEYDIG CELL STEROIDOGENESIS

In the testes, Leydig cell steroidogenesis is dependent on both acute and chronic stimulation by the anterior pituitary hormone, luteinizing hormone (LH) (Cooke *et al.*, 1976; Dufau *et al.*, 1971; Moyle & Ramachondran, 1973; Purvis *et al.*, 1981; van der Molen & Rommerts, 1981; Ewing *et al.*, 1983). LH stimulation of testosterone synthesis and secretion is initiated by LH binding to high affinity receptors on the surface of Leydig cells (Catt *et al.*, 1972). The binding of LH to its receptor results in increased production of intracellular cyclic adenosine 3',5'-monophosphate (cAMP) (Dufau *et al.*, 1973). Both the acute and the chronic effects of LH are mediated by increases in cAMP. The acute effect of LH or cAMP is to increase the transport of cholesterol to the inner mitochondrial membrane where cholesterol associates with the cholesterol side chain cleavage enzyme ($P\text{-}450_{scc}$), which catalyses the cleavage of the side chain of cholesterol to yield the C_{21} steroid, pregnenolone. The production of C_{19} steroid, testosterone from C_{21} precursors requires the activities of cytochrome $P\text{-}450_{17\alpha}$ ($P\text{-}450_{17\alpha}$), which is associated with the smooth endoplasmic reticulum. $P\text{-}450\text{-}_{17\alpha}$ catalyzes two reactions, the

hydroxylation of the C_{21} steroids, progesterone or pregnenolone (17α-hydroxylase activity), followed by cleavage of the two-carbon side chain (C_{17-20} lyase activity) to yield the C19 steroid androstendione or dehydroepiandrosterone, respectively (Fevold *et al.*, 1989). Androstendione is the immediate precursor of testosterone. This latter reaction is catalyzed by 17-ketosteroid reductase, a non P-450 enzyme (Bogovich & Payne, 1980).

Chronic stimulation by LH or cAMP is required for optimal synthesis of the P-450 enzymes involved in testosterone biosynthesis. Chronic treatment of intact or hypophysectomized rats with LH or its analogue, human chorionic gonadotrophin (hCG), results in increased capacity for LH-stimulated testosterone production (Zipf *et al.*, 1978; Payne *et al.*, 1980), and induce enzymes of the steroidogenic pathways (Purvis *et al.*, 1973a; Purvis *et al.*, 1973b; O'Shaughnessy & Payne, 1982). In contrast, administration of a single high dose of LH or hCG to intact animals results in a decreased capacity for LH-stimulated testosterone production and in a decrease in $P450_{17\alpha}$ activities (O'Shaughenessy & Payne, 1982) and reduced amount of testicular mitochondrial P-450 (Luketich *et al.*, 1983).

1.3.1. Sources of cholesterol

The Leydig cell is the main site of androgen production (Cooke *et al.*, 1972; van der Molen & Rommerts, 1981). In Leydig cells as in other steroidogenic tissues such as the adrenals, ovary, and placenta, cholesterol is used as a precursor during steroidogenesis. Cholesterol used for steroidogenesis can be obtained by three different mechanisms in steroidogenic cells: (1) uptake of

plasma lipoprotein particles, (2) *de novo* synthesis from acetate, and (3) hydrolysis of cholesteryl esters stored in lipid droplets.

1.3.1.1. Uptake of plasma lipoprotein particles

Chen *et al.* (1980) were the first to report specific binding of ^{125}I-rat high density lipoprotein (HDL) to isolated interstitial cells of rat testis. An increase in the number of low density lipoprotein (LDL)/HDL binding sites resulting from treatment with LH/ or hCG *in vivo* and *in vitro* has been demonstrated in testicular cells. In studies using testicular fractions, Chen *et al.* (1980) reported that treatment of rats with hCG for 4 days caused 2.2 fold increase in rat HDL binding to membranes of the interstitial tissue fraction with no effect on binding affinity. Benahmed *et al.* (1981) demonstrated that the addition of hCG to cultures of porcine Leydig cells increased the number of LDL binding sites per Leydig cell between 1.5 and 2 fold after 24 h of incubation. However, Leydig cells of the testis, do not appear to be as dependent on exogenous cholesterol under normal physiological conditions as adrenal or ovarian cells (Ascoli, 1981; Quinn *et al.*, 1981; Hou *et al.*, 1990). The utilization of lipoprotein particles as a source of cholesterol becomes important only after severe depletion of intracellular pool due to prolonged stimulation with gonadotrophins either *in vivo* or *in vitro* (Quinn et al., 1981; Freeman & Ascoli, 1982; Benahmed *et al.*, 1983).

1.3.1.2. *De novo* synthesis of cholesterol

It is generally accepted that biosynthesis of cholesterol from acetate occurs in the SER and involves the microsomal enzyme 3-hydroxy-3-methylglutaryl-

CoA (HMG-CoA) reductase (Tsai *et al.*, 1964; Scallen & Sanghvi, 1983; Hardie *et al.*, 1989). However, Pignataro *et al.* (1983) have demonstrated that cholesterol can be synthesized inside the mitochondria in rat Leydig cells. The HMG-CoA reductase is tightly regulated: high levels of cholesterol decreases the synthesis of mRNA and increases the degradation of this protein. The membrane domain of this enzyme was found to be crucial for the regulation by cholesterol, since deletion of this domain by site-directed mutagenesis produced in transfected cells a soluble form of the enzyme that is perfectly functional in cholesterol synthesis, but whose degradation is no longer stimulated by extracellular cholesterol (Hardie *et al.*, 1989). Another mechanism that has been proposed for the regulation of HMG-CoA reductase is the reversible phosphorylation of the enzyme, phosphorylation catalyzed by HMG-CoA reductase kinase (AMP-activated protein kinase) inactivates the enzyme, while dephosphorylation catalyzed by phosphoprotein phosphatase I activates HMG-CoA reductase (Scallen & Sanghvi, 1983; Hardie *et al.*, 1989). Studies on the mechanism of regulation of HMG-CoA reductase in gonadal cells are limited. Studies in cultured mouse tumour Leydig cells (MA10) indicate that, in the absence of exogenous source of cholesterol, when steroid hormone production by these Leydig cells is stimulated with hCG, HMG-CoA reductase activity increases 3 fold during the first 4h and then decline to normal levels during the next 8h followed by a further decline (50-70% of control) between 24 and 72h after hCG treatment (Charreau *et al.*, 1981; Freeman & Ascoli, 1982). The acute increase in HMG-CoA reductase activity was suggested to be

a result of intracellular cholesterol depletion, since this can be inhibited by aminoglutethimide (an inhibitor of cholesterol metabolism) (Freeman & Ascoli, 1982; Hou *et al.*, 1990). However, unlike adrenal and ovarian cells, lipoprotein-derived cholesterol supply does not seem to affect the activity of HMG-CoA reductase in Leydig cells (Charreau *et al.*, 1981; Freeman & Ascoli, 1982). In spite of the increase in HMG-CoA activity in mouse Leydig cells incubated *in vitro* with hCG, lovastatin, a potent inhibitor of this enzyme, had no effect on unstimulated or hCG-stimulated testosterone production during a 12h incubation (Hou *et al.*, 1990).

1.3.1.3. Intracellular storage of cholesterol

Intracellular cholesterol can be stored as cholesteryl esters in lipid droplets. Two enzymes are directly related to the amount of cholesterol stored as cholesteryl esters; Acyl-coenzyme A: cholesterol acyltransferase (ACAT) and cholesteryl ester hydrolases (CEH). ACAT is associated with SER and catalyses the esterification of cholesterol is associated with the SER while CEH are present predominantly in the cytosol and catalyse the hydrolysis of cholesteryl esters. The storage of cholesteryl esters as lipid droplets in Leydig cells varies among species and also during sexual maturation in the rat. Leydig cells from immature rats contain many more lipid droplets than do Leydig cells from mature rats (Payne *et al.*, 1982). Normally, 95% or more of the cholesterol in the rat testis is present in an unesterified form (Pearlman, 1950; van der Molen et al., 1972), and newly synthesized cholesterol from acetate mixes with the pool(s) of free cholesterol. However, in mouse testis 10 to 40% of the

cholesterol occurs in an esterified form (Bartke, 1971; Pokel *et al.*, 1972; Hou et al., 1990). In spite of the low levels of cholesterol esters in the rat Leydig cells, cholesteryl ester hydrolases (CEH) are present in the developing rat testis. Total specific activity of CEH has been found to be constant from 14 to 47 days, increased by as much as two fold by 51 to 150 days (Wee & Grogan, 1989). The temperature stable CEH has been found in both Leydig cells and Sertoli cells, and this was greatly reduced by hypophysectomy and induced by LH and FSH in Leydig cells and Sertoli cells respectively. The temperature labile CEH has been found in Sertoli cells, and this was undetectable in hypophysectomized rats and stimulated by FSH but not LH (Durham & Grogan, 1982; Durham & Grogan, 1984). Cholesteryl ester hydrolase is also present in M5480 murine Leydig cell tumours and can be modulated by hCG (Albert *et al.*, 1980). Bailey & Grogan (1986), Colbran *et al.* (1986), and Wee & Grogan (1989) showed that CEH were activated by cAMP-dependent protein kinase and inhibited reversibly by phosphatase. It was suggested that activation of Leydig cell CEH by LH/hCG is one of the intracellular mechanisms for providing cholesterol for steroidogenesis (Pokel *et al.*, 1972; Albert *et al.* 1980; Wee & Grogan, 1989).

1.3.1.4. Plasma membrane storage of cholesterol

Studies using murine Leydig tumour cells (MA10 cells) indicate that the plasma membrane is the major source of cholesterol used for steroidogenesis during acute steroid response (Freeman & Ascoli, 1982; Freeman, 1987, 1989). The plasma membrane cholesterol enters the cell by internalization of the plasma

membrane (Nagy & Freeman, 1990). Newly synthesized cholesterol, cholesterol ester-derived cholesterol, and lipoprotein-derived cholesterol are cycled through the plasma membrane before its use for steroidogenesis (Nagy & Freeman, 1990). The cholesterol is transported to the plasma membrane via vesicles (Kaplan & Simoni, 1985; 1989). However, not all cholesterol used for steroid biosynthesis passes through the plasma membrane, diversion of newly synthesized and lipoprotein-derived cholesterol has been detected in MA10 cells (Freeman, 1987).

1.3.2. Mobilization of cholesterol

LH stimulation acutely increases the mobilization of cholesterol from the cytoplasm to the mitochondria, the transport of free cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, and transport and redistribution of cholesterol in the inner mitochondrial membrane, thereby facilitating the binding of cholesterol to the active site of cytochrome P-450-dependent side-chain cleavage complex ($P\text{-}450_{scc}$) on the inner face of the inner mitochondrial membrane, and the cleavage of the side chain of cholesterol. Studies in adrenal (Crivello & Jefcoate, 1980; Vahouny *et al.*, 1983), Leydig (Hall *et al.*, 1981), and luteal (Toaff *et al.*, 1979) cells show that the major acute effect of stimulation of these cells with cAMP or trophic hormone is an increase in the transport of cholesterol to the mitochondrial $P\text{-}450_{scc}$ which involves several factors.

1.3.2.1. Role of *de novo* protein synthesis

Several investigators have reported that a small molecular weight protein obtained from cytosol or mitochondria mediates the transport of cholesterol (Bakker *et al.*, 1978; Lefèvre *et al.*, 1978). In addition it has been shown that the acute regulation of steroidogenesis requires *de novo* synthesis of proteins, as demonstrated in studies employing protein synthesis inhibitors (Arthur & Boyd, 1974; Cooke *et al.*, 1975; Mendelson *et al.*, 1975; Cooke *et al.*, 1979). The inhibition in steroidogenesis produced by cycloheximide, a protein synthesis inhibitor, appears to be related to the redistribution of cholesterol within the mitochondria, which makes the substrate available to the active site of P-450_{scc}. Privalle *et al.* (1983) demonstrated that cycloheximide blocks the transport of cholesterol from the mitochondria outer membrane to the inner membrane, thus pinpointing the site of action of the 'regulatory' protein. Since cholesterol would tend to exchange very slowly between membranes separated by an aqueous phase, such as the intermembrane space of the mitochondria, it has been proposed that trophic hormone action could result in the accelerated transport of cholesterol from outer to inner membrane through increased contacts between the two membranes (Stevens *et al.*, 1985). Many efforts have been made to identify the short lived protein(s) which may be responsible for this stimulation. Several studies have attempted to correlate the appearance of newly synthesized proteins with the observed increase in steroid production in various tissues. These studies resulted in the identification of proteins in adrenal, granulosa and testicular cells and whose synthesis is

concommitant with the increased production of steroids (Pederson & Brownie, 1987; Stocco & Kilgore, 1988; Yanagibashi *et al.*, 1988).

To date three structurally different proteins which appear to perform the same *in vitro* function have been proposed as 'regulatory' proteins. These are sterol carrier protein-2 (SCP_2), steroidogenesis activator polypeptide (SAP), and "8.2K" protein (Strott, 1990).

1.3.2.1.1. Sterol carrier protein-2 (SCP_2)

In the rat testis, SCP_2 is specifically localized in the Leydig cells (van Noort *et al.*, 1986). The amount of SCP_2 in the cytosol of Leydig cells and in adrenocortical cells is under hormonal control (van Noort *et al.* 1986; 1988b; Trzeciak *et al.*, 1987; Mendis-Handagama *et al.*, 1990), suggesting the involvement of SCP_2 in the hormonal control of steroidogenesis. Addition of LH to rat tumour Leydig cells results in a two fold increase in SCP2 levels in the cytosol which is sustained for at least 24h (van Noort *et al.* 1988b.

1.3.2.1.2. Steroidogenesis activator polypeptide (SAP)

SAP was first isolated from a rat H-540 Leydig cell tumour (Pederson & Brownie, 1987). The mechanism by which SAP stimulates cholesterol side-chain cleavage activity is not understood, but it has been suggested to facilitate the intramitochondrial distribution of cholesterol (Pederson & Brownie, 1987).

1.3.2.1.3. "8.2 k" protein

The "8.2k" protein (PI= 6.5 and Mr 8200) was isolated from bovine adrenocortical cells (Yanagibashi *et al.*, 1988). The "8.2K" protein directly stimulates $P\text{-}450_{scc}$ activity in isolated adrenocortical mitochondria in a dose

dependent fashion; it also facilitates the translocation of cholesterol from the outer to the inner mitochondrial membrane, and promoted the binding of substrate to the cholesterol side-chain cleavage P-450 (Yanagibashi *et al.*, 1988). The 8.2K protein was found to be identical (except that it lacks two residues at the carboxyl-terminus) to a brain protein called endozepine (diazepam binding inhibitor, DBI), which inhibits the binding of diazepam to benzodiazepine/$GABA_A$ receptors (Besman *et al.*, 1989). Recently, a precursor of endozapin (DBI or "8.2 K"protein), octadecaneuropeptide (ODN) was localized in rat Leydig cells by immunocytochemistry and *in situ* hybridization, suggesting that this peptide is produced and stored in Leydig cells. The presence of peripheral benzodiazepine receptors (PBDR) and ODN in Leydig cells is suggestive of a role of ODN and/or other DBI-related peptides in the regulation of Leydig cell steroidogenesis. More direct evidence for a role of benzodiazepines in testicular function has been obtained by *in vitro* studies on whole decapsulated testis or crude interstitial cells (Wilkinson *et al.*, 1980; Ritta *et al.*, 1987), and purified rat Leydig cells and mouse tumour Leydig cells (MA10) (Papadopoulos *et al.*, 1990). Papadopoulos *et al.* (1990) have clearly demonstrated that PBDR are implicated in the acute stimulation of Leydig cell steroidogenesis possibly by mediating the entry, distribution and/or availability of cholesterol within the mitochondria. In both MA10 cells and rat Leydig cells, the most potent PBDR-ligands stimulated steroid production. Unlike hCG or cAMP-stimulated steroidogenesis, the stimulation by the ligands was not inhibited by cycloheximide and was not additive to that of hCG,

cAMP or 22R-hydroxycholesterol. PBDR-ligands stimulated in a dose dependent manner pregnenolone biosynthesis by isolated mitochondria when supplied with exogenous cholesterol, and this effect was not observed in mitochondria devoid of the outer membrane. A similar stimulatory effect of benzodiazepines on steroidogenesis was demonstrated in differentiated granulosa primary cultures and in granulosa cells (Amsterdam & Suh, 1991).

1.3.3. Conversion of cholesterol to pregnenolone by P-450$_{scc}$

1.3.3.1. Components of the enzyme system and their interactions

The rate limiting enzymatic step in the synthesis of steroids by steroidogenic tissue is the side chain cleavage of cholesterol to form pregnenolone and isocaproaldehyde (Drosdowsky *et al.*, 1965; Chaudhary & Stocco, 1988; Rouiller *et al.*, 1990). This reaction is catalysed by a mini electron transport system known as the cholesterol side chain cleavage complex (CSCC) which is located on the matrix side of the inner mitochondrial membrane (Lambeth *et al.*, 1987; Privalle *et al.*, 1987; Yanagibashi *et al.*, 1988). Cytochrome P-450$_{scc}$ binds cholesterol and catalyses sequential hydroxylations at carbon 22 and 20 followed by cleavage of the C_{20}-C_{22} to yield pregnenolone and isocaproaldehyde (Figure 1.2). The reaction requires 3 mol of NADPH and 3 mol of molecular oxygen (Chaudhary & Stocco, 1988; Miller, 1988). Protein purification studies and *in vitro* reconstitution of enzymatic activity showed that a single protein termed P-450$_{scc}$ is responsible for all the steps between cholesterol and pregnenolone (Hall *et.*, 1979; Shikira & Hall, 1973a,b; Simpson, 1979) on a single active site (Duque *et al.*, 1978). This was confirmed by protein

purification data which suggest the existence of a single immunologically identifiable species of P-450$_{scc}$ in a multimeric form of 16 subunits totaling over 850 kD (Shikira & Hall, 1973a,b). Human and bovine P-450$_{scc}$ share 82% amino acid homology and 71% nucleotide homology. The testicular enzyme appears to have similar properties to the adrenal and ovarian enzymes (Bakker *et al.*, 1978; Perkins & Payne, 1988).

As predicted from the southern blotting data showing the presence of a single human P-450$_{scc}$ gene, the human adrenal and testicular P450$_{scc}$ cDNA clones have identical sequences (Chung *et al.*, 1987) and the unique human gene encoding the P-450$_{scc}$ was localized on chromosome 15 (Chung *et al.*, 1986). In the rat, P-450$_{scc}$ can be detected in Leydig cells from 15.5 day old male foetuses and ovaries from 8.5 day old female neonates (Rouiller *et al.*, 1990). P-450$_{scc}$ receives electrons from an iron/ sulfur protein termed adrenodoxin, which is found in solution in the mitochondrial matrix (Suzuki & Kimura, 1965; Kimura & Suzuki, 1967). Adrenodoxin in turn receives electrons from a flavoprotein that is loosely bound to the inner mitochondrial membrane (Kimura & Sizuki, 1965). This flavoprotein termed adrenodoxin reductase, receives electrons from NADPH. Virtually all the information available on the interactions of the components of the side-chain cleavage enzyme complex are from studies of the adrenal enzyme. The binding of cholesterol to P-450$_{scc}$ enhances the binding of adrenodoxin to P-450$_{scc}$ by about 20 fold, and the binding of adrenodoxin enhances the binding of cholesterol to the P-450$_{scc}$. The binding of reaction intermediates, 22R or 20α-hydroxycholesterol or 22R, 20α-

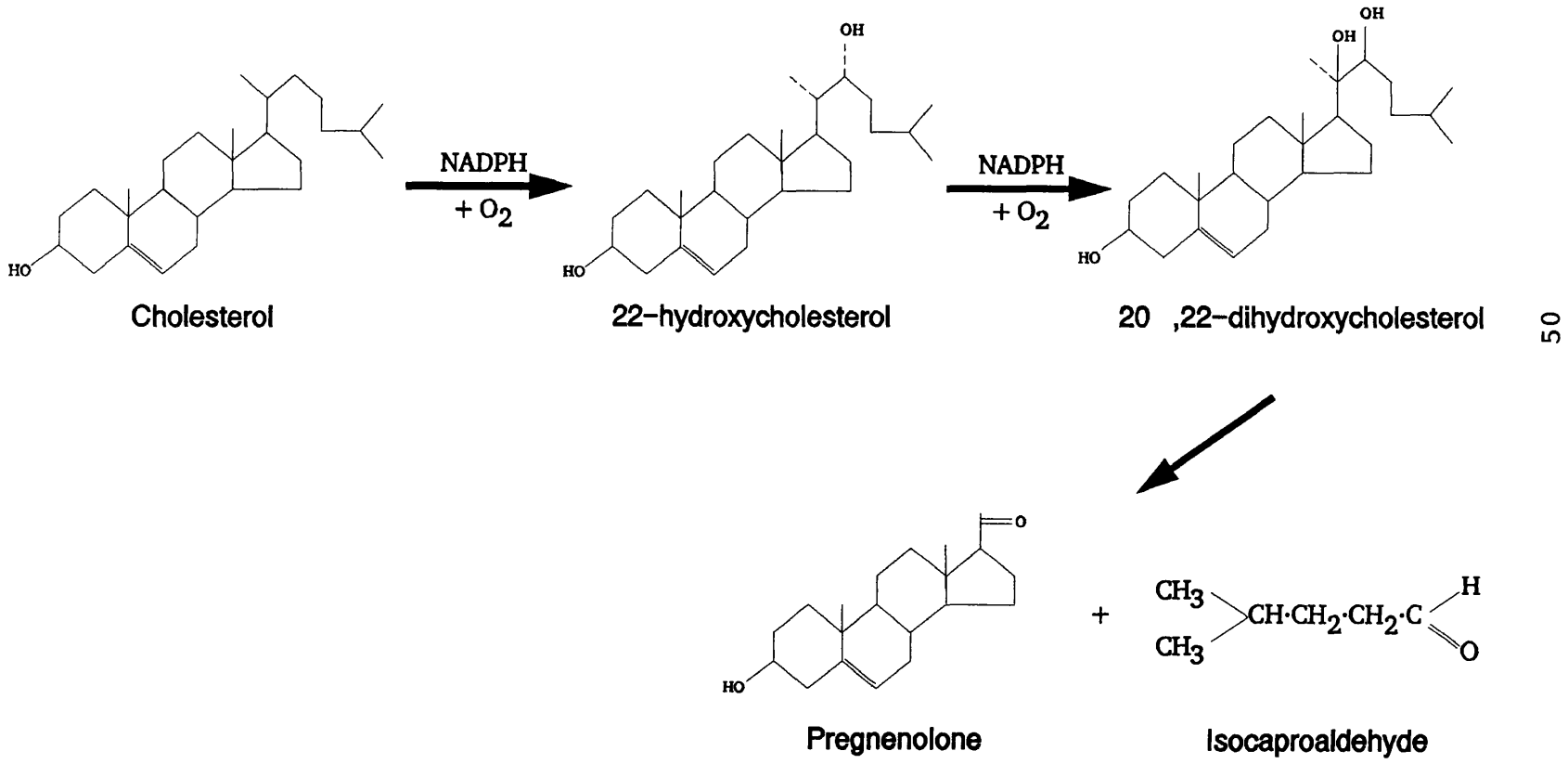
dihydrocholesterol, is 100 to 300 times tighter than that of cholesterol. This prevents competition from substrate or product and ensure that, once cholesterol is bound, complete hydroxylation is virtually inevitable (Lambeth *et al.*, 1982).

1.3.3.2. Metabolisms of hydroxysterols

Based on studies on the metabolism of hydroxycholesterol, the key regulatory step in the mechanism of trophic hormone-stimulated steroid production was suggested to be at the first hydroxylation step (at C_{22}) of the 3 sequential monoxygenation reactions involved in the conversion of cholesterol to pregnenolone (Chaudhary & Stocco, 1988).

The hydroxylated sterols bind more tightly to P-450_{scc} than cholesterol and are metabolized to pregnenolone more rapidly than cholesterol. Rat Leydig cells are more responsive to 22R, 20, or 25-hydroxycholesterol than to cAMP, with 22R-hydroxycholesterol being more potent than 20 or 25-hydroxycholesterol (Quinn *et al.*, 1985). When both cholesterol and 20- or 25-hydroxycholesterol are present in the incubation medium, pregnenolone production rates are mainly influenced by the hydroxycholesterol, even in the presence of a ten fold excess of cholesterol (Bakker *et al.*, 1979).

Figure 1.2. Possible intermediates in the formation of pregnenolone from cholesterol (Gower, 1988).



The conversion of 20-, 25- or 22R-hydroxycholesterol to testosterone (rat and mouse Leydig cells) or progesterone (MA10 Leydig cells) was found to be cycloheximide insensitive (Bakker *et al.*, 1979; Quinn *et al.*, 1985; Chaudhary & Stocco, 1988), not affected by LH/hCG or cAMP (Quinn *et al.*, 1985; Chaudhary & Stocco, 1988), insensitive to Ca^{+2} (Bakker *et al.*, 1979), and independent of the microfilament-mediated transport process (Quinn *et al.*, 1985). There is species-specific difference in the control of mitochondria cholesterol metabolism between the rat and mouse, metabolism of 25-hydroxycholesterol by Leydig cells from out- or inbred mice (Quinn *et al.*, 1985) and MA10 Leydig cells (Chaudhary & Stocco, 1988) yields significantly less testosterone and progesterone respectively than that produced in responce to cAMP. This suggests that there are differences between mice and rats in P-450_{scc}, cholesterol transport, or in the regulatory factors controlling the side chain cleavage of cholesterol.

1.3.3.3. Regulation of P-450_{scc}

P-450_{scc} is known to be an important site of acute and chronic regulation by trophic hormones (Waterman & Simpson, 1985). Menon *et al.* (1967) first showed that side chain cleavage activity is under control of gonadotrophins. Hypophysectomy decreased this activity considerably in the rat testis and subsequent gonadotrophin treatment returned the activity to near normal levels (Purvis *et al.*, 1973ab; O'Shaughnessy & Payne, 1982). Anderson & Mendelson (1984) have shown that gonadotrophin specifically induces synthesis of P-450_{scc} in rat Leydig cells. Induction of $P450_{scc}$ by trophic hormones has also been demonstrated for granulosa cells (Toaff *et al.*, 1983; Simpson *et al.*, 1988; Ook *et al.*, 1989; Urban *et al.*, 1990) and

adrenocortical cells (Jefcoate *et al.*, 1974; John *et al.*, 1986; Simpson *et al.*, 1988).

There is no evidence that LH acutely stimulate the P-450$_{scc}$ in any steroidogenic tissue. The induction of P-450$_{scc}$ by trophic hormones was demonstrated to be through cAMP, since the latter mimicks the effect of the trophic hormones (Voutilainen *et al.*, 1986; Goldring *et al.*, 1987; Voutilainen & Miller, 1987; Simpson *et al.*, 1988; Hales & Payne, 1989; Oonk *et al.*, 1989). Masson *et al.* (1984) showed that treatment of immature porcine testicular cells in culture with hCG increases *de novo* synthesis of P-450$_{scc}$. Studies of rat Leydig cells in culture by Anderson & Mendelson (1985) demonstrated that treatment with hCG or dcAMP increases the synthesis of P-450$_{scc}$ and an associated protein, iron sulphur protein (adrenodoxin), which is involved in the transport of electrons from NADPH to P-450$_{scc}$. Orava *et al.* (1989) showed that treatment of immature porcine Leydig cells with hCG results in an increase in P-450$_{scc}$ mRNA. The regulation of P-450$_{scc}$ in mouse Leydig cells and mouse tumour Leydig cells (MA10) is described fully in a review by Payne (1990), P450$_{scc}$ was found to be constitutively expressed in both cell types. Chronic cAMP stimulation increases the steady state levels of P-450$_{scc}$ mRNA and *de novo* P-450$_{scc}$ protein synthesis. This stimulation is achieved with a very low concentration of cAMP (10μM 8-bromo-cAMP) which has no significant effect on steroidogenesis.

In addition to trophic hormones, other factors can modify the expression of P-450$_{scc}$, most of which do not operate via increase in the levels of cAMP. Insulin-like growth factor (IGFI) and epidermal growth factor (EGF) can

stimulate $P450_{scc}$ expression by granulosa cells, and Leydig cells, and other agents such as phorbol esters are inhibitory of cAMP-stimulated expression of $P\text{-}450_{scc}$ (Ascoli *et al.*, 1987; Simpson et al., 1988; Verhoeven & Cailleau, 1988; Urban *et al.*, 1990; Simpson *et al.*, 1990). In addition, glucocorticoids, specifically, decrease the constitutive and cAMP-induced synthesis of $P450_{scc}$ protein as well as the accumulation of $P450_{scc}$ mRNA (Hales & Payne, 1989).

1.3.4. Metabolism of pregnenolone

Once synthesized, pregnenolone is further metabolized to the active steroids, C_{21}-progestins, C_{19}-androgens, and C_{18}-oestrogens (Figure 1.3) (van der Molen & Rommerts, 1981; Johnson & Everitt, 1984; Payne *et al*, 1985, Gower, 1988). Pregnenolone is metabolized to testosterone in Leydig cells of the testis and the theca cells of the ovary by enzymes associated with the smooth endoplasmic reticulum 3ß-hydroxysteroid dehydrogenase-isomerase, 17α-hydroxylase, C_{17-20} lyase, and 17-ketosteroid reductase. In the testis, testosterone can be metabolized further to oestrogens or 5α-reduced androgens by Leydig cells or Sertoli cells. The production of pregnenolone is acutely as well as chronically regulated by LH. Although the enzymes of the steroidogenic pathway(s) distal to pregnenolone production are generally not under acute regulation by LH, LH is important in the tonic regulation of pregnenolone metabolism (Payne *et al.*, 1985). In addition LH can act alone or in concert with prolactin, FSH, and gonadal steroids to regulate pregnenolone metabolism (Payne *et al.*, 1985).

1.3.4.1. 3ß-Hydroxysteroid dehydrogenase-isomerase (3ß-HSD)

The conversion of 3ß-hydroxysteroids to 3-ketosteroids is catalyzed by the enzyme complex, 3ß-hydroxysteroid dehydrogenase and 3-ketosteroid isomerase (3ß-HSD). These reactions are poorly characterized and their enzymatic bases poorly understood. The oxidized form of nicotinamide adenine dinucleotide (NAD^+) is required as an acceptor for the 3ß-HSD activity. Recent protein purification data suggest the presence of a single 3ß-HSD/isomerase protein (Ishi-Ohba *et al.*, 1986) although very early enzymatic studies suggested the presence of two or three different isozymes of the isomerase specific for the three different steroidogenic pathways (mineralocorticoid, glucocorticoid, and sex steroids). In the rat and the mouse, pregnenolone is the preferred substrate for 3ß-hydroxysteroid dehydrogenase-isomerase and the 3-ketosteroid pathway is preferred (Samuels *et al.*, 1975), whereas in the human, pig, bovine, the 3ß-hydroxysteroid pathway appears to be the predominant (Neher & Wettstein, 1960; Yanaihara & Troën, 1972; Fevold *et al.*, 1989). Leydig cell 3ß-HSD is under chronic regulation by LH as demonstrated by many investigators. Using the whole testes, it was shown that a single injection of hCG increased the 3ß-HSD activity in the testis of both immature (Shikita & Hall, 1967) and mature (Samuels & Helmreich, 1956) rats within 48h of treatment. Also repeated injections with LH/hCG resulted in an increase in 3ß-HSD in hypophysectomized mature rats (Shikira & Hall, 1967; Shaw *et al.*, 1979). In subsequent studies, the effects of both single and repeated injections with LH or hCG have been studied using isolated interstitial tissue or purified Leydig

cells. No effect on 3ß-HSD activity in testicular interstitial tissue could be found 48h after adult rats were given a single s.c. injection of up to 100 IU hCG (Nozu *et al.*, 1981).

1.3.4.2. 17α-hydroxylase and C_{17-20} lyase ($P\text{-}450_{17\alpha}$)

Both pregnenolone and progesterone may undergo 17α-hydroxylation to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. The latter 17α-hydroxylated steroids may then undergo scission of the C_{17-20} carbon bond to yield dehydroisoandrosterone and androstendione respectively (Fevold *et al.*, 1989). It is now well demonstrated that all four of these reactions are mediated by a single enzyme, $P\text{-}450_{17\alpha}$. This $P\text{-}450_{17\alpha}$ is bound to smooth endoplasmic reticulum (Shikira & Tamaoki, 1965) and requires a flavoprotein immunologically distinct from mitochondrial adrenodoxin which is involved in the transfer of electrons from NADPH to cytochrome P-450 (Baron *et al.*, 1972; Betz *et al.*, 1976). According to studies in the pig Leydig cell (Nakajin & Hall, 1981; Nakajin *et al.*, 1981) and the guinea pig (Kominami *et al.*, 1982), the pig and bovine (Zuber *et al.*, 1986) adrenal glands, 17α-hydroxylase and C_{17-20} lyase activity appear to be associated with a single purified cytochrome P-450. Unlike $P\text{-}450_{scc}$ which is expressed in all steroidogenic tissues, the expression of $P\text{-}450_{17\alpha}$ shows tissue specificity in some species. $P\text{-}450_{17\alpha}$ is not expressed in the rat (Voutilainen & Miller, 1986; Nishihara *et al.*, 1988) or in the mouse (Perkins & Payne, 1988) adrenal cortex or in the MA10 tumour Leydig cells (Payne, 1990).

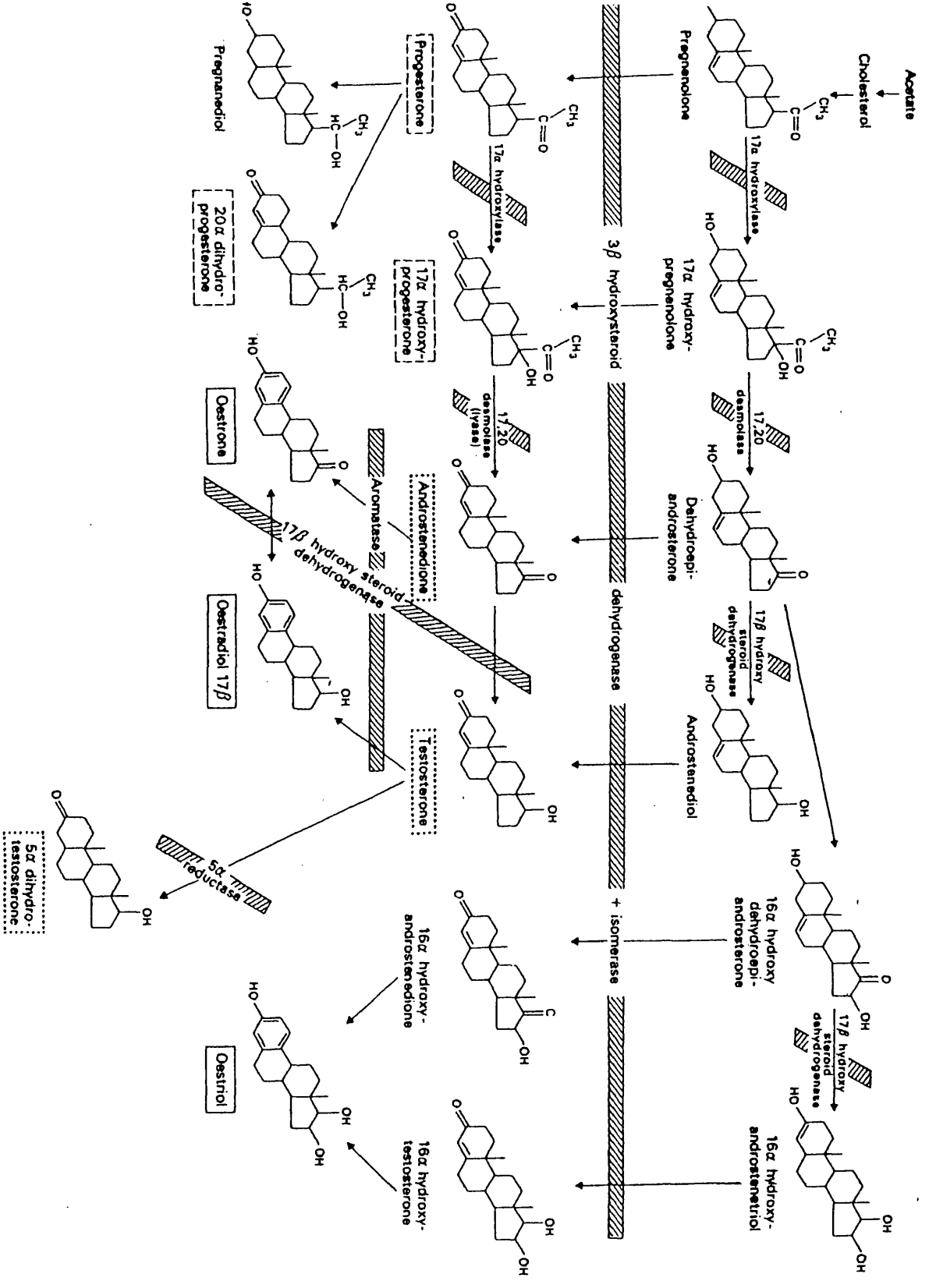


Figure 1.3. Pathways involved in the biosynthesis of testicular steroids from cholesterol (Johnson & Everitt, 1984).

It appeared that the pig has two isozymes of P-$450_{17\alpha}$, one expressed in the adrenal and the other in the testis (Nakajin & Hall, 1981; Nakajin *et al.*, 1984) while in human, the same gene is expressed in both tissues (Chung *et al.*, 1987). In the rat testis P-$450_{17\alpha}$ activity was found to be highest in the Leydig cell fraction, but is also detectable in the non-Leydig cell fraction of interstitial cells as well as in the seminiferous tubules (Kühn *et al.*, 1983). In spite of a limited importance of P-$450_{17\alpha}$ in the regulation of steroidogenesis demonstrated by Anakwe & Payne (1987) and Perkins & Payne (1988), there is considerable evidence to support the role of LH in the regulation of P-$450_{17\alpha}$. Hypophysectomy of adult rats results in a specific decrease in 17α-hydroxylase and C_{17-20} lyase with a half life of 2.3 and 3.4 days, respectively, and this effect is prevented with subsequent chronic treatment of the hypophysectomized animals for 4 to 8 days (Purvis *et al.*, 1973a). Testicular interstitial cells from adult rats treated with 50 or 100 IU hCG 48h prior to killing had reduced 17α-hydroxylase and C_{17-20} lyase activities (Nozu *et al.*, 1981). A single high dose of hCG or LH results in a decrease in subsequent hCG-stimulated testosterone production (Nozu *et al.*, 1981; O-Shaughnessy & Payne, 1982). However, there is increasing evidence that the decrease in 17α-hydroxylase and C_{17-20} lyase observed during steroidogenic desensitization is not the primary cause of the subsequent decrease in hCG-stimulated testosterone production (Quinn *et al.*, 1981; Quinn & Payne, 1984).

In addition to LH, locally produced growth factors are involved in the regulation of the expression and activity of P-$450_{17\alpha}$. Using ovine

adrenocortical cells, Rainey *et al.* (1988) have demonstrated that the chronic inhibitory effect of TGFß on steroidogenesis involves a decrease in the activity and amount of P-$450_{17\alpha}$ protein associated with a decrease in cAMP production. A similar effect of TGFß on steroidogenesis was shown in both rat (Lin *et al.*, 1987) and porcine (Avallet *et al.*, 1987; 1988). In contrast the effects of TGFß on another steroidogenic tissue, ovarian granulosa cells, are mainly stimulatory (Adashi & Resnick, 1986; Knecht *et al.*, 1986; Dodson & Chomberg, 1987; Adashi *et al.*, 1989), while no effect was demonstrated in theca cells (Adashi *et al.*, 1989. Fibroblast growth factor (FGF) was also found to cause inhibition of P-$450_{17\alpha}$ in rat (Fauser *et al.*, 1988) and porcine (Raeside *et al.*, 1988) Leydig cells. On the other hand, EGF was found to inhibit P-$450_{17\alpha}$ in primary cultures of crude rat testicular cells incubated for several days in the absence of LH (Welsh & Hsueh, 1982), while a stimulatory effect of EGF on steroidogenesis was demonstrated in freshly isolated rat and mouse Leydig cells (Verhoeven & Cailleau, 1986), and in mouse tumour Leydig cells (MA10) (Ascoli *et al.*, 1987).

1.3.4.3. 17-Ketosteroid reductase and 17ß-hydroxysteroid dehydrogenase

The microsomal enzyme, 17-ketosteroid reductase (17-KSR), catalyses the conversion of the 17-ketosteroids, dehydroepiandrosterone and androstendione, to the corresponding 17ß-hydroxysteroids, androstene-3ß,17ß-diol and testosterone respectively, in the presence of NADPH. The reverse reaction is catalyzed by 17ß-hydroxysteroid dehydrogenase in the presence of $NADP^+$. Murono and Payne (1976) reported two distinct 17-ketosteroid reductase

enzymes in rat testes, one in the interstitial tissue and one in the seminiferous tubules with pH optima of 5.6 and 6.5 respectively. At the optimum pH, the apparent Km for androstendione is 17μM for the interstitial enzyme and 0.25 μM for the tubular enzyme. Testosterone competitively inhibits the interstitial enzyme but appears to stimulate the 17-KSR activity of the seminiferous tubules (Murono & Payne, 1976). There is evidence that testicular 17-KSR is under pituitary hormone control. Treatment of adult intact rats with a single injection of 50 or 100IU hCG has no effect on 17-KSR activity in testicular interstial tissue 48h after treatment (Nozu *et al.*, 1981). However, using purified Leydig cells, O'Shaughnessy and Payne, (1982) have shown that injection of intact adult rats with 5IU hCG/day for 6 days does not alter 17-KSR activity in Leydig cell population IA and IB, but decreases by 63% the activity of Leydig cell population II. The regulation of Leydig cell 17-KSR by pituitary hormones is strengthened by the observation by Nozu *et al*, (1981) showing that 3 days posthypophysectomy, 17-KSR activity is decreased by 50% in interstial cells from adult rat testes.

1.3.4.4. 5α-reductase

5α-reductase is found principally associated with the smooth endoplasmic reticulum (Forchielli & Dorfman, 1956; Moore & Wilson, 1972), although, there have been reports of 5α-reductase in nuclear preparations (Moore & Wilson, 1972). The major pathway of testosterone metabolism in rat testis is via 5α-reductase. The activity of 5α-reductase is age dependent, during sexual maturation, 5α-reductase activity is high before 35 to 40 days of age (Ficher

& Steinberger, 1971; Tsujimura & Matsumoto, 1979; Moger, 1979). Testicular 5α-reductase activity has been demonstrated in both the interstitium and the seminiferous tubules (Folman *et al.*, 1973; Yoshizaki *et al.*, 1978; Rivarols & Podesta, 1972; Rivarols *et al.*, 1972; Dorrington & Fritz, 1975). The existence of 5α-reductase in the Leydig cell of immature animals is consistent with reports that various treatment with LH or hCG can enhance the enzyme activity in testicular tissue (Shikita & Hall, 1967; Murono & Payne, 1979; Purvis *et al.*, 1973). However, since chronic treatment with hCG can increase the number of Leydig cells per testis (Chemes *et al.*, 1976; Christensen & Peacock, 1980), the increase in testicular 5α-reductase may be due, at least in part, to an increase in the number of Leydig cells. This is supported by a study by O'Shaughnessy & Payne (1982), in which treatment of intact adult rats with hCG for 6 days resulted in a decrease in 5α-reductase activity in Leydig cell population 1A and no change in activity in Leydig cell population 1B and II.

On the other hand, a mouse Leydig cell tumour line (T124958-R), showed an increase in 5α-reductase activity in response to *in vivo* or *in vitro* treatment with oestrogens whereas hCG had no significant effect on this enzyme.

1.3.4.5. Aromatase

1.3..4.5.1. Structure and cellular locatization of aromatase

Aromatase is the enzyme responsible for the conversion of androgens to oestrogens. The enzyme complex is localized in the endoplasmic reticulum of cells in which it is expressed, and consists of two components (Thompson &

Siiteri, 1974a). The first is a form of cytochrome P-450 known as aromatase cytochrome P-450 ($P\text{-}450_{AROM}$). This heme protein is a glycoprotein with three potential N-linked glycosylation sites (Sethumadhavan *et al.*, 1991) and is responsible for binding the C_{19} steroid substrate and catalyzing the series of reactions leading to the formation of a phenolic A ring. The second is a flavoprotein, NADPH: cytochrome P-450 reductase which is responsible for transferring reducing equivalents from NADPH to cytochrome P-450. Since there is only one gene encoding the reductase, this enzyme must be capable of transferring reducing equivalents to any form of microsomal cytochrome P-450 that it encounters (Simmons *et al.*, 1985).

1.3.4.5.2. Mechanism of aromatization

As shown in Figure 1.4., the aromatase reaction requires 3 mol of molecular oxygen and 3 mol of NADPH per mol of C_{19} steroid metabolized (Thompson & Siiteri, 1974b). The first two oxygen molecules are used in the oxidation of the C_{19} angular methyl group and the products of the two hydroxylations are 19-hydroxyandrostenedione and 19-oxoandrostenedione (Meyer, 1955; Morato *et al.*, 1961), whereas the site of attack of the third molecule of oxygen is uncertain. It has been suggested that the third site of oxygen attack is at the 2ß position with a subsequent nonenzymic collapse of the 2ß-hydroxy-19-aldehyde to oestrogen (Goto & Fishman, 1977; Fishman & Raju, 1981; Hahn & Fishman, 1984); however, more recent work suggests that peroxidative attack, again involving the C_{19} methyl group, may be involved instead (Cole & Robinson, 1988).

Figure 1.4. Aromatase reaction sequence.

The conversion of androgens to oestrogens requires the consumption of 3 molecules of NADPH and O_2 for each molecule of oestrogen formed. NADPH provides the reducing equivalents while oxygen is inserted into the molecule by three sequential hydroxylations. The first hydroxylation takes place at the C19 position to generate the C19 alcohol. The second hydroxylation is also at the C19 position, resulting in the C19 aldehyde. The final hydroxylation may take place at the C19 position resulting in the loss of the C19 methyl group as formic acid (HCOOH) and the 1ß-hydrogen is stereospecifically transfered to water (Lephart & Simpson, 1991).

O
H*
O
O
O2
NADPH
HO
H*
O
O
OH
O
O2
NADPH
O
H*
HO
OH
O
O2
NADPH
O
H*
HO
OH
O
H2O
HO
O
HCOOH
HOH*
-OH*

In the last step, the 1ß-hydrogen is stereospecifically transfered to water (Fishman & Raju, 1981), and the C_{19} fragment is expelled as formic acid (Townsley & Brodie, 1968; Brodie *et al.*, 1969; Akhtar *et al.*, 1982; Caspi *et al.*, 1984; Cole & Robinson, 1988). The issue whether the three reactions are accomplished by one or more catalytic sites has as yet not been resolved, with arguments being presented for either a single (Kelly *et al.*, 1977) or a multiple site system (Fishman & Goto, 1981). Based on kinetic properties of cytochrome $P\text{-}450_{AROM}$ expressed in COS1 cells, Corbin *et al.* (1988) and Mendelson *et al.* (1990) demonstrated the presence of only one aromatase enzyme in human, capable of aromatizing all three substrates namely androstenedione, testosterone, and 16α-hydroxyandrostendione, to the corresponding products, namely oestrone, oestradiol, and 16α-hydroxyoestrone.

1.3.4.5.3. Tissue distribution of aromatase

The aromatization reaction occurs in a number of cells and tissues including ovarian granulosa cells (McNatty *et al.*, 1976; Erickson *et al.*, 1989), placenta (Fournet-Dulguerov *et al.*, 1987; Osawa *et al.*, 1987; Chen *et al*, 1988; Nestler, 1990), testicular Sertoli (Dorrington & Armstrong, 1975; Fritz et al., 1976; Khan & Nieschlag, 1991) and Leydig cells (Valladares & Payne, 1979ab; Tsai-Morris *et al.*, 1984; Papadopoulos *et al.*, 1986), adipose tissue of both male and females (Grodin *et al.*, 1973; Ackerman *et al.*, 1981; Nimrod & Ryan, 1975; Edman & MacDonald, 1978), various sites of brain including the hypothalamus (Roselli *et al.*, 1985), human genital skin fibroblasts (Berkovitz *et al.*, 1984; Fujimoto *et al.* 1986; Stillman *et al.*, 1990; Berkovitz *et al.*, 1990),

hepatocytes (Lanoux *et al.*, 1985), the preimplantation blastocyst (Brodie *et al.*, 1977), as well as in tumour cells or tissues (Pierrepoint *et al.*, 1966; Pattilo *et al.*, 1972; Bercovici *et al.*, 1981; Tilson-Mallett *et al.*, 1983, Orczyk *et al.*, 1987, Lephart *et al.*, 1990).

The capacity of testicular tissue to synthesize oestrogens has been known for many years. The secretion of oestradiol by human testis was first demonstrated by Kelch *et al.* (1972) and then subsequently confirmed by Baird *et al.* (1973) and Payne *et al.* (1976). In the rat and in man, the amount of oestradiol secreted by the testis constitutes about 20% of the circulating levels of this steroid (de Jong, 1974).

1.3.4.5.1. Regulation of aromatase activity and/or synthesis

The evidence for the regulation of oestrogen biosynthesis by LH/hCG was first demonstrated by Maddock & Nelson (1952) who showed an increase in urinary excretion of oestrogens after administration of hCG to men. This was subsequently confirmed by Weinstein *et al.* (1974) in man and by de Jong *et al.* (1973), Valladares & Payne (1979a), and Pomerantz (1981) in the rat. There was a considerable controversy as to the intratesticular site of aromatization and as to which gonadotrophin regulates testicular aromatization. *In vitro* studies by de Jong *et al.* (1974) suggested that oestrogens are synthesized in seminiferous tubules and not in interstitial tissue of adult rat testis. A study by Benahmed *et al.* (1982) also excluded the Leydig cell contribution to testicular aromatization in immature pig testes. Dorrington & Armstrong (1979) reported that Sertoli cells obtained from immature rats synthesize oestradiol

when maintained in culture in the presence of follicle-stimulating hormone FSH and testosterone. The presence of aromatase activity in Sertoli cells of immature rats and its regulation by FSH and cAMP was demonstrated in recent studies by Weniger & Zeis (1988) and Khan & Nieschlag (1991). A study by Tsai-Morris *et al.* (1985) shows that both Sertoli cells and Leydig cells have the capacity for aromatization, with the Leydig cells being the major site for aromatization in the rat testis. This study was confirmed by other studies using purified Leydig cells (Valladares & Payne, 1979b; Papadopoulos et al., 1986; Orczyk *et al.*, 1987) and also by a study showing an excessive oestrogen production by Leydig cell tumours (Pierrpoint *et al.*, 1966).

The levels of oestradiol produced by isolated Leydig cells in response to hCG *in vitro* was found to be variable, it ranges from 17% (Orczyk *et al.*, 1987), 30% (Tsai-Morris *et* al., 1985), 2 fold (Papadopoulos *et al.*, 1986), to 8 fold increase (Valladares & Payne, 1979b). The effect of hCG was found to be mediated by cAMP, since dcAMP mimicked the effect of hCG (Valladares & Payne, 1979b; Papadopoulos *et al.*, 1986). In addition to LH/hCG, prolactin and dexamethasone were also found to affect aromatase activity in isolated rat Leydig cells. They both induce a 20% increase in the basal production of oestradiol, but inhibit by 45% oestradiol production in response to LH/hCG or dcAMP. However, a transplantable rat Leydig cell tumour LTW(m) (Mordes *et al.*, 1984), which was found to be associated with high circulating levels of oestrogen and low levels of testosterone, was found to have an active autonomous aromatase system that is not responsive to compounds affecting

the adenylate cyclase/cAMP system nor to phorbol esters (Orczyk *et al.*, 1987). A rat Leydig cell tumour cell line (R2C) exhibits a high level expression of aromatase activity, in contrast to the mouse tumour Leydig cell line (MA10) with no detectable levels of this enzyme (Lephart *et al.*, 1990). The R2C Leydig tumour cells were found to exhibit a different mode of regulation by the factors controling aromatase activity compared with the normal rat Leydig cells. The high level expression of aromatase in these cells was found not to be affected by dexamethasone, but was inhibited with forskolin or 8-bromo-cAMP and EGF (Lephart *et al.*, 1990). The complexity of the regulation of oestrogen biosynthesis is also shown in otheir tissues. In adipose stromal cells, aromatase activity is stimulated by glucocorticoids (Simpson *et al.*, 1981, 1989) as well as by cAMP analogues (Mendelson *et al.*, 1982). Stimulation by the latter is inhibited by growth factors, such as EGF, PDGF, FGF, TGFß, TGFα, TNF, and IL-1ß, but is markedly potentiated by phorbol esters (Mendelson *et al.*, 1986; 1987, Simpson *et al.*, 1989). Similarly, in human skin fibroblasts, dexamethasone was found to cause an increase in aromatase activity which can be inhibited by actinomycin D and cycloheximide (Fujimoto *et al.*, 1986; Stillman *et al.*, 1990). Also f$\overset{o}{\underset{\wedge}{e}}$tal hepatocytes show a similar mode of regulation of aromatase activity to that in adipose stromal cells, dexamethasone, cAMP & cholera toxin stimulate aromatase activity (Lanoux *et al.*, 1985). In the ovary, in addition to FSH which is a major stimulus of aromatase activity (Erickson & Hsueh, 1978), IGFI was found to have a stimulatory effect on the induction of aromatase and to act in synergy with

FSH and LH to maintain maximal levels of oestradiol synthesis by these cells (Erickson *et al.*, 1989). In Sertoli cells from immature rats, IL1ß was found to inhibit FSH-stimulated aromatase activity (Khan & Nieschlag, 1991). In addition to gonadotrophins, growth factors and cytokines, 5α reduced androgen metabolites, namely, androsterone, 5α-androstenedione and dihydrotestosterone were found to be potent inhibitors of aromatase activity in breast carcinoma cell lines (Perel *et al.*, 1984; 1988). Testosterone also was found to exhibit an inhibitory effect in human skin fibroblasts (Berkovitz *et al.*, 1990).

In spite of the differences in the regulation of aromatase activity in different tissues and cell types, 4-hydroxy-androstendione was found to be a universal inhibitor (Brodie *et al.*, 1976; 1977; Johnston & Metcalf, 1984; Brandt *et al.*, 1988; Miller, 1989; Stillman *et al.*, 1990) (Figure 1.5). Using polyclonal and monoclonal antibodies specific for aromatase cyt P-450, it was demonstrated that the action of the various factors on the regulation of aromatase activity in adipose and ovarian tissue is due to alterations in the rate of aromatase cyt P-450, whereas the synthesis of the reductase component of the aromatase enzyme complex was relatively unaffected (Mendelson & Simpson, 1987; Mendelson *et al.*, 1987). The changes in the rate of synthesis of aromatase cyt P-450 was associated with changes in the levels of translatable mRNA specific for this protein (Evans *et al.*, 1987; Mendelson *et al.*, 1987; 1990). The increase in cyt $P\text{-}450_{AROM}$ mRNA induced by cAMP and phorbol ester are apparently mediated by a regulatory protein(s), similar to that found for other steroidogenic forms of cyt P-450 (Evans *et al.*, 1987).

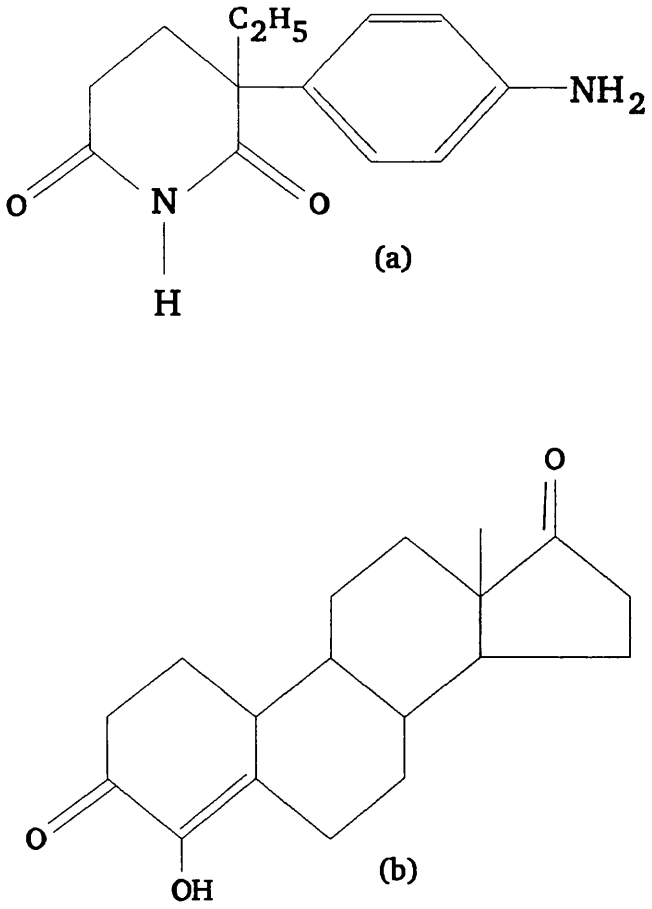


Figure 1.5. The chemical structure of two aromatase inhibitors; amino-glutethimide (a) and 4-hydroxyandrostenedione (4OHA) (Lønning *et al.*, 1990).

1.3.4.6. Oestradiol-17ß hydroxysteroid dehydrogenase

Oestradiol-17ß hydroxysteroid dehydrogenase (E2-17ß HSD) is involved in the synthesis of oestradiol. It also has a pivotal role in regulating tissue concentrations of this biologically active oestrogen, mediating the conversion of oestrone to oestrodiol or metabolism of oestradiol to oestrone.

This enzyme is detectable in the testis, ovary, and other tissues including the placenta, endometrium, lung, prostate and breast (Reed, 1990; Mann *et al.*, 1991). Based upon measurement of enzyme activity, multiple forms of E2-17ß HSD have been reported (Blomquist *et al.*, 1987; Mann *et al.*, 1991). However, different forms of the enzyme have not yet been sequenced or cloned, and it is not yet known whether the same or different enzymes act as oestrogen and androgen oxidoreductases. Tremblay *et al.* (1989) suggested that the oxidoreductase of androgens may be mediated by a different enzyme, since the mRNA for E2-17ß HSD (1.4 kb) was not always detectable in tissues in which androstendione is converted to testosterone.

The control of E2-17ß HSD activity is still unknown. In endometrial tissues during the secretory phase of the menstrual cycle, there is convincing evidence that the progesterone-induced increase in E2-17ß HSD activity acts in an oxidative (i.e. converts oestrodiol to oestrone) direction (Schmidt-Gollwitzer *et al.*, 1979). In breast tissue, the conversion of oestrone to oestradiol was found to be predominant, thus accounting for increased concentrations of oestradiol that are found in breast tumours (for refs, see Reed, 1991).

1.4. REGULATION OF LEYDIG CELL FUNCTION

1.4.1. Hypothalamic and Pituitary Control

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are synthesized in the anterior pituitary gonadotrophs and act on the testis and ovary to direct steroidogenesis and gametogenesis. The gonadotrophs are heterogeneous with respect to their storage pattern for LH and FSH (Childs *et al.*, 1983). The relative proportions of the two main gonadotroph subtypes, monohormonal (LH) and multihormonal (LH/FSH), vary depending on the endocrine status of the animal. Exposure to GnRH causes a shift in the gonadotroph population from LH and LH/FSH cells to LH/FSH cells (Childs *et al.*, 1982; Lloyds & Childs, 1988; Garner *et al.*, 1990).

1.4.1.1. Structure of Luteinizing hormone and human chorionic gonadotrophin

The two gonadotrophic hormones, LH and FSH, are members of the glycoprotein family of hormones which also includes the pituitary hormone thyroid-stimulating hormone (TSH) and placental chorionic gonadotrophin (hCG). The glycoprotein hormones are a family of heterodimeric hormones that share a common 92-amino acid α-subunit encoded by a single gene which is linked to hormone-specific ß-subunits (Pierce & Parsons, 1981; Gordon & Ward, 1985; Wallis *et al.*, 1985; Catt & Pierce, 1986; Ryan *et al.*, 1988; Erickson *et al.*, 1990). Both subunits of LH/hCG contribute to the biological activity (Strickland & Puett, 1981). Although the ß subunits determine biological specificity of the hormones, there is a high degree of amino acid homology between these subunits (Stewart & Stewart, 1977), which is most apparent for LHß and hCGß.

They are 85% homologous in the first 114 amino acids (Talmadge *et al.*, 1984), and this relationship is responsible for the binding of hCG and LH to a common gonadal receptor (Pierce & Parsons, 1981; Wallis *et al.*, 1985; Catt & Pierce, 1986). Because of the greater availability of hCG and because of its slower dissociation rate from the LH/hCG receptor (which makes radioligand assays easier) and stability, hCG is the hormone usually used experimentally to study the LH/hCG receptor (Salesse *et al.*, 1983; Cooke & Rommerts, 1988; Segaloff *et al.*, 1990).

1.4.1.2. The role of carbohydrate moities in LH/hCG function

Studies based on chemical and enzymatical modifications of carbohydrate moieties in hCG have revealed that the terminal sialic acid residues are important in prolonging the plasma circulating life of the hormone, and that successive exoglycosidase treatments of asialo-hCG reduce its ability to stimulate the production of cAMP and steroids in rat Leydig cells (Moyle, 1975; Cooke *et al.*, 1990) and mouse tumour MA10 cells (Sairam, 1990). Although glycoprotein hormone binding to plasma membrane receptors involves a discontinuous site on the hormone that spans both the α and ß subunits (see Ryan *et al.*, 1988 for a review) and both α- and ß-subunits are involved in hormone action (Keutmann *et al.*, 1983), the carbohydrate structures in the ß-subunit play a dominant role in the determination of the biological activity (Shimohigashi & Chen, 1982). Since the impaired adenylate cyclase activity is associated with a normal membrane receptor binding in rat Leydig cells (Moyle, 1975; Keutmann *et al.*, 1983) and enhanced binding activity in MA10

cells (Sairam, 1990) and human granulosa-luteal and Leydig cells (Ranta *et al.*, 1987), it was suggested that the carbohydrate moieties for LH/hCG may be involved in receptor/adenylate cyclase coupling.

1.4.1.3. The hypothalamic control of pituitary gonadotrophins

Although many parts of the brain may influence reproductive function, the hypothalamus can be considered as the final common pathway through which the control of gonadotrophin secretion and behaviour are mediated. LH and FSH synthesis and secretion are regulated by the decapeptide gonadotrophin releasing hormone (GnRH) (Figure 1.6) which is released from the median eminence of the hypothalamus in a pulsatile manner (see Clayton, 1987 for a review). After synthesis in the perikarya of hypothalamic neurons, GnRH is transported along axons to be stored in the nerve terminals of the median eminence region of the hypothalamus. GnRH release into hypophysial-portal capillaries is affected by neurotransmitters of the central catecholaminergic system, originating in many brain areas (Fink, 1979) and by endogenous opioids (EOPs) (Blank *et al.*, 1986). The administration of GnRH stimulates the release of both LH and FSH from the gonadotrophs by a calcium-dependent mechanism (Borges *et al.*, 1983; Clapper & Conn, 1985; Catt & Stojilkovic, 1989; Dan-Cohen & Naor, 1990; Davidson *et al.*, 1991). In addition to the native hormone, many potent agonists (Kolho & Huhtaniemi, 1989) and antagonists (Puente & Catt, 1986) of GnRH have been synthesized. These synthetic agonists and antagonists of GnRH not only are useful for study of the physiology of the pituitary gonadal axis, but also are potential agents for infertility and fertility

control (Corbin & Bex, 1980; Borgman *et al.*, 1982; Faure *et al.*, 1982; Redding *et al.*, 1982; Skarin *et al.*, 1982; Donald *et al.*, 1983; Waxman *et al.*, 1987). Studies in several mammalian species including the rat (Haisenleder *et al.*, 1991), primate (Marshall & Kelsh, 1986), and sheep (McIntosh & McIntosh, 1985) have shown that a pulsatile GnRH signal is required to maintain gonadotrophin secretory activity, because a continuous GnRH infusion desensitizes the gonadotroph cell to further GnRH stimulation and down regulates GnRH receptors (Clayton, 1982). The pattern (amplitude and frequency) of the GnRH pulsatile signal has been shown to change in various reproductive states. For example, GnRH pulse frequency increases during the follicular phase of the rat oestrous (Fox & Smith, 1985) and human menstrual cycles (Reame *et al.*, 1984; Marshall & Kelsh, 1986). Both pulse frequency and amplitude increase during preovulatory LH and FSH surge in the rat and in women (Reame *et al.*, 1984; Fox & Smith, 1985). Other studies have shown that GnRH pulse frequency and amplitude increase during pubertal maturation in rats and human (Marshall & Kelch, 1986; Bourguignon & Frachimont, 1984) and after gonadectomy (Urbanski *et al.*, 1988). In addition to effects on secretion, GnRH also regulates the synthesis of pituitary gonadotrophins. Studies in rats and mice using GnRH agonist/antagonist administration or GnRH antisera have demonstrated the role of GnRH in maintaning gonadotrophin subunit mRNA levels (Saade *et al.*, 1989; Wierman *et al.*, 1989; McNeilly *et al.*, 1991). Using a castrate and testosterone replaced rat model in which endogenous GnRH is suppressed, it was shown that both GnRH pulse frequency and amplitude regulate the synthesis of

gonadotrophin subunits (Dalkin *et al.*, 1989). Studies in rats (Papavasiliou *et al.*, 1986; Dalkin *et al.*, 1989; Haisenleder *et al.*, 1991) and sheep (Hamernick & Nett, 1988) have revealed that the nature of the pulsatile GnRH signal can selectively regulate gonadotrophin subunit gene transcription with faster frequencies increasing α and LHß and slower pulses FSHß (Haisenleder *et al.*, 1991; Rosen *et al.*, 1991). A reduction in LH synthesis and release in response to LHRH agonists occurs with ageing, the defect being manifested at middle age (Béliste *et al.*, 1991).

The effects of GnRH on pituitary gonadotrophs are mediated by GnRH receptors (Ban *et al.*, 1990). GnRH was found to have a biphasic autoregulatory effect on its own pituitary receptors; up-regulation is consistently observed with low dose continuous infusion of native GnRH and GnRH agonists while net loss of GnRH receptors is produced by continuous exposure to high agonist concentrations (Clayton *et al.*, 1982; Ban *et al.*, 1990).

Although GnRH is the major regulator of LH secretion, several other neuromediators exert direct modulation of gonadotroph function at the pituitary level. These include opiate (Blank *et al.*, 1986), oxytocin, vasopressin (Evans *et al.*, 1989), neuropeptide-Y (Crowley *et al.*, 1987; Kaynard *et al..*, 1991; Parker *et al.*, 1991) and the gamma-aminobutyric-acid (GABA) (Virmani *et al.*, 1990).

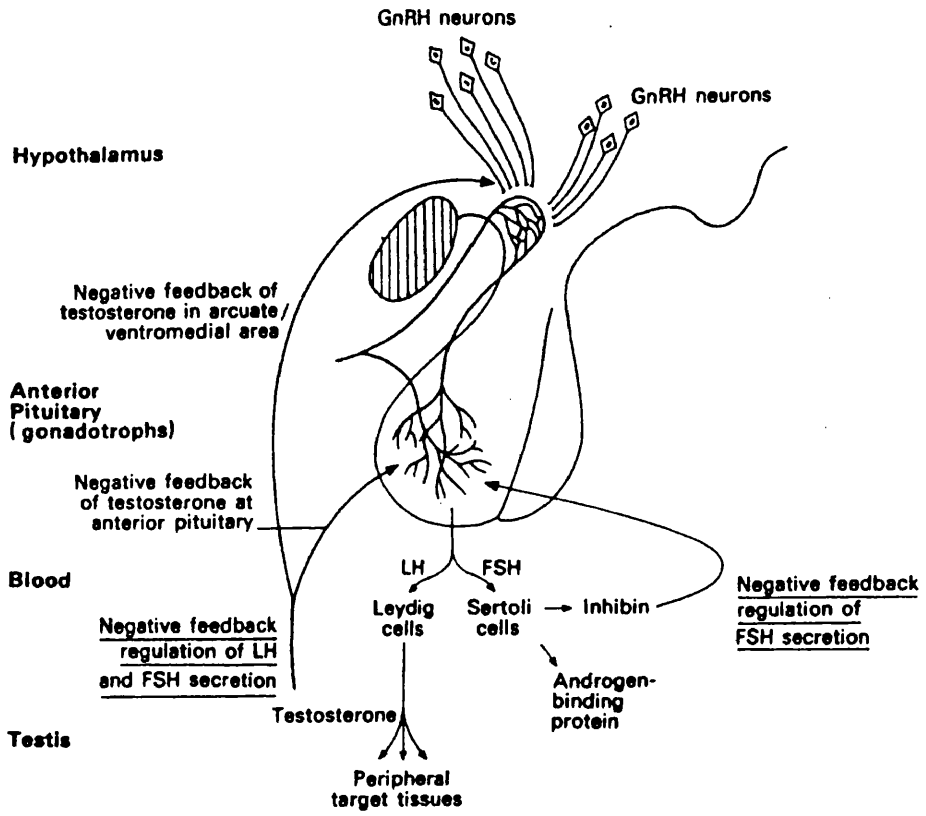


Figure 1.6. Diagrammatic summary of the regulation of FSH and LH secretion in the male (Johnson & Everitt, 1984).

1.4.1.4. The control of pituitary gonadotrophins by gonadal peptides

The gonadal peptides inhibin, activin and follistatin were isolated based on their effects on FSH secretion from the pituitary gland. The related peptides inhibin and activin have opposite effects on FSH, with the αß ($\alpha\beta_A$ and $\alpha\beta_B$) heterodimer, inhibin, suppressing FSH secretion (Robertson *et al.*, 1985), and the ßß (A ($\beta_A\beta_A$) and AB ($\beta_A\beta_B$) homodimer, activin, stimulating FSH release (Ling *et al.*, 1986). Both peptides have been identified in the gonads (Merchenthaler *et al.*, 1987; Lee *et al.*, 1989; Risbridger *et al.*, 1989; Shaha *et al.*, 1989), and mRNA for the α and β_B subunits of inhibin have been localised in the pituitary (Meunier *et al.*, 1988, Houben & Denef, 1990; Corrigan *et al.*, 1991). The mechanism by which inhibin exert its effect on FSH secretion is not clear, an effect on GnRH receptors was demonstrated; Braden *et al.* (1990) have reported that inhibin prevents the up-regulation of GnRH receptors by blocking stimulation of GnRH receptor synthesis by GnRH. In addition, analysis of FSH pulse parameters have indicated that inhibin interferes with pulse frequency, amplitude, and peak levels of FSH, an effect suggested to be not entirely mediated by changes in GnRH receptors (Rivier *et al.*, 1991). On the other hand activin was found to increase the number of follicle-stimulating hormone cells in anterior pituitary cultures (Katayama *et al.*, 1990, 1991).

1.4.1.5. The regulation of pituitary gonadotrophins by gonadal steroids

In males the serum levels of gonadotrophic hormones are under classical negative feedback control from gonadal steroids. The onset of negative-feedback regulation of gonadotrophin synthesis by gonads and/or gonadal steroids starts at 7 days of age in male rats (Pakarinen & Huhtaniemi, 1989). However, in females, oestrogen feedback regulation of LH is both negative and positive. Rising serum oestrogen concentrations from the maturing ovarian follicle sensitize the gonadotroph to GnRH stimulation, with resulting augmentation of LH release during mid-cycle (Clayton, 1987). The way by which sex steroids regulate synthesis of gonadotrophins is very complex. Gonadectomy results in an increase in mRNA levels for all three gonadotrophin subunits in male and female rats (Gharib *et al.*, 1987). In addition *in situ* hybridization studies have shown that both the number of LHß and FSHß mRNA-containing cells and the amount of LHß and FSHß mRNA per cell increase following gonadectomy (Childs *et al.*, 1990). In male rats, testosterone regulates the pattern of gonadotrophin secretion by acting at several levels of the hypothalamic-pituitary axis. Not only does testosterone modify the responsiveness of the pituitary gonadotrophs to GnRH (Kamel *et al.*, 1987; Strobl *et al.*, 1989; Culler, 1990) but it also regulates both *in vitro* (Kalra *et al.*, 1987; Welsel *et al.*, 1989) and *in vivo* (Dluzen & Ramirez, 1987) GnRH release from the hypothalamus. Testosterone exerts a differential effect on FSH and LH secretion, the effect on FSH being less pronounced compared with that on LH (Kotsuji *et al.*, 1988; Kitahara *et al.*, 1990). The inhibitory effect

of testosterone on LH secretion is firmly established, but its effect on GnRH synthesis and release is not clear. Studies on changes of hypothalamic GnRH mRNA following gonadectomy have obtained contradictory results (Wiemann *et al.*, 1990; Selmanoff *et al.*, 1991). Similarly, observations of the effect of testosterone on GnRH secretion in castrated male rats also have given puzzling results. Testosterone treatment *in vivo* does not suppress GnRH content of the hypothalamus nor does it suppress GnRH release from hypothalamic tissue incubated *in vitro* (see, Kalra & Kalra, 1983 for a review). Indeed there is evidence that testosterone may increase mRNA for GnRH precursor in the hypothalamus (Park *et al.*, 1988; Selmanoff *et al.*, 1991) and thus increase hypothalamic GnRH content (Roselli *et al.*, 1990). Many of these observations suggest that testosterone stimulates rather than inhibits GnRH secretion, an idea contrary to the concept of testosterone inhibiting gonadotrophin secretion by inhibiting both the hypothalamus and the pituitary. The role and effect of testosterone may differ among species; in the rat (Steiner *et al.*, 1982) and in the ram (Jackson *et al.*, 1991), testosterone was found to reduce GnRH pulse frequency.

However, in human, testosterone and/or its metabolites inhibit LH and FSH secretion by a GnRH-independent mechanism (Sheckler *et al.*, 1989).

At the pituitary level, testosterone was found to exert an inhibitory effect on LH subunit gene expression. It also has additional inhibitory actions on translational and postranslational events in gonadotrophs (Abbot *et al.*, 1988).

The stimulatory effects by testosterone and oestradiol on GnRH in the hypothalamus may involve neuropeptide Y (NPY), since both steroids stimulate hypothalamic NPY levels and release and there is anatomical evidence of colocalization which is suggestive of a direct genomic modulation of NPY neurosecretion by steroids in a subpopulation of hypothalamic NPY neurons (Sahu *et al.*, 1990; Sar *et al.*, 1990)

1.4.2. The adrenal control (glucocorticoids)

The early observation by Selye (1939) that stress is accompanied by both an increase in the activity of the hypothalamic pituitary-adrenal axis (HPA) and a decrease in reproductive functions had suggested a possible relationship between hormones of the HPA and those of the hypothalamic pituitary-gonadal axis (HPG). Several investigators have shown that under a range of physiological and pathological conditions, excess cortisol/ corticosterone has profound effects on the male reproductive system (Gabrilove *et al.*, 1979; Doerr & Pirke, 1976, 1979; Saez *et al.*, 1977; Schaison *et al.*, 1978; Purvis & Hansson, 1978; McKenna *et al.*, 1979; Cumming *et al.*, 1983; Mann *et al.*, 1987). These studies demonstrated an association of elevated blood concentrations of either endogenous or exogenous glucocorticoids with decreased testicular testosterone production. In Cushing's syndrome, men often show decreased libido or impotence, decreased testosterone, and pathological changes in the seminiferous tubules, which return to normal after bilateral adrenalectomy (McKenna *et al.*, 1979).

The glucocorticoid-induced inhibition of testicular function may occur at the hypothalamic, hypophyseal or testicular level. Several investigators have shown that glucocorticoids modify the secretion of LH in various species including rats, bulls, and man (Baldwin & Sawyer, 1974; Krulich *et al.*, 1974; Sakakura *et al.*, 1975; Chantoraprateep & Thibier, 1978; Du Rmissean *et al.*, 1978; Gray *et al.*, 1978; Barb *et al.*, 1982). Glucocorticoids block basal LH secretion by rat pituitary cells (Suter & Schwartz, 1985) and the GnRH-induced secretion of LH in rat (Rosen *et al.*, 1991) and bovine (Padmanabhan *et al.*, 1983) pituitary cells. Similar effects have been observed *in vivo* in male (Ringstrom & Schwartz, 1985) and female (Baldwin, 1979) gonadectomized rats and in the rhesus monkey (Dubey & Plant, 1985). However, patients with Cushing disease were found to have normal basal and GnRH-stimulated gonadotrophin levels in the presence of suppressed testosterone (McKenna *et al.*, 1979).

The inhibitory effect of glucocorticoids on post-castration rise in gonadotrophins was found to be mediated through opioids, since the opioid antagonists have the ability to reverse the glucocorticoid-inhibiting effect on LH (Belhadj *et al.*, 1989).

The direct effect of glucocorticoids on testicular testosterone secretion is supported by many studies (Desjardins & Ewing, 1971; Saez *et al.*, 1977; Bambino & Hseuh, 1981). A major part of the activity of glucocorticoids is believed to be dependent on the decreased production of arachidonic acid and its metabolites by inhibition of the action of the membrane-bound phospholipase A2 (PLA_2). This inibitory effect, which requires RNA and protein

synthesis (Irvine, 1982), was found to be mediated by lipocortins (Flower, 1988). Little is known on the mechanism of action of lipocortins, but some evidence indicates that inhibition of PLA_2 activity by these proteins is due to binding and sequestration of the substrate, rather than a specific effect on the enzyme (for Refs, see Whitehouse, 1989). In addition glucocorticoids can also affect arachidonic acid availability in the long term by its effect on the enzymes involved in the synthesis of arachidonic acid from linoleic acid and by an increase in its esterification (Irvine, 1982). In addition, there is evidence that glucocorticoids have a direct inhibitory effect on steroidogenic enzymes, an effect mediated by glucocorticoid receptors (Ortlip *et al*, 1981). *In vitro* studies by Hale & Payne (1989) showed a decrease in the constitutive and cAMP-induced synthesis of $P450_{scc}$ protein as well as the accumulation of $P450_{scc}$ mRNA in mouse Leydig cells. In addition, a decreased 17α-hydroxylase activity in rat Leydig cells was reported by Welsh *et al.* (1982).

The mechanism by which chronic exposure to excess exogenous corticosteroids or to physiological levels of glucocorticoids affects testicular function is not understood. It was suggested that the degree of exposure of the testis to active glucocorticoid is in part controlled by local metabolism of the steroid by 11ß-hydroxysteroid dehydrogenase (11ß-HSD) which catalyses the oxidation of cortisol and corticosterone to their inactive metabolites, cortisone and 11-dehydrocorticosterone in human and the rat respectively (Phillips *et al.*, 1989). Gene expression and activity of 11ß-HSD have been demonstrated in various tissues of the rat including the testis (Krozowski *et al.*, 1990; Monder &

Lakshmi, 1990). Immunofluorescence studies by Phillips *et al.* (1989) are suggestive of the localization of this enzyme in the Leydig cells, since its appearance was found to correlate temporally with the postnatal increase in Leydig cell number and serum testosterone (Phillips *et al.*, 1989).

1.4.3. The intratesticular control

It is generally accepted that the development and function of the testis are dependent on the gonadotrophic hormones secreted by the anterior pituitary. However, it is becoming increasingly evident that the function of the testis is also locally controlled. The gonadotrophic hormones including LH, FSH and to a lesser extent prolactin, provide the basic stimuli on which testicular function depends. The effect(s) of the stimuli appears to be determined by complex intratesticular factors which coordinate many different events that comprise the over-all function of the testis. The importance of the intratesticular control in the regulation of testis function was suggested to occur: 1) to modulate Leydig cell responsiveness to LH according to local requirements; 2) to prevent intratesticular levels of testosterone from falling below a particular level during the interval between successive LH pulses (i.e. when LH levels are low); 3) to control differentially the intratesticular and peripheral effects of testosterone (e.g. during early puberty when high local concentrations of testosterone are required to drive spermatogenesis while the levels in the periphery are undetectable) (Sharpe, 1990). Cell-cell interaction in the testis has been a subject of many studies (Sharpe, 1983, 1984, 1986, 1990; Skinner, 1991 for reviews). The majority of research on the cell biology

of the testis has focused on Sertoli, peritubular myoid, Leydig, and developing germinal cells. Interactions with other cell types including macrophages, lymphocytes, endothelial, and mast cells have also been recently reported.

1.4.3.1. Leydig cell-peritubular cell interactions

The interaction between Leydig cells and peritubular myoid cells was found to be mediated by androgens. The peritubular cells are the target for androgens (Nakhla *et al.*, 1984; Anthony *et al.*, 1989). Isolated peritubular cells in culture respond to androgens, e.g. the production of testicular paracrine factor PModS was found to be under androgen control (Skinner & Fritz, 1985a; Skinner & Fritz, 1985b). This factor appears to be important for the maintenance of testis function and the process of spermatogenesis (Skinner *et al.*, 1988; Norton & Skinner, 1989). Based on these observations, it was postulated that a cascade of cellular interactions occur in response to LH. LH acts on the Leydig cells to stimulate the production of androgen that subsequently act on the peritubular cells to stimulate the production of PModS. In addition, Peritubular cells secrete other factors that may influence Leydig cell function include insulin-like growth factor (IGF-I) (Cailleau *et al.*, 1990), epidermal growth factor (EGF)/ transforming growth factor-α (TGFα), and -ß (TGFß) (Skinner *et al.*, 1989).

1.4.3.2. Leydig cell-macrophage interactions

Functional interactions exist between testicular macrophages and Leydig cells; cultured macrophages from adult rats have been shown to bind and respond to FSH by secreting a factor(s) that stimulates testosterone secretion by cultured Leydig cells (Yee & Hutson, 1983; 1985a,b). This phenomenon was found to be specific for testicular macrophages, since peritoneal macrophages are unable to respond to FSH (Yee & Hutson, 1985a). The other evidence for the expression of a tissue specific phenotype by testicular macrophages is based on their difference in the protein profile compared with peritoneal macrophages as studied by two dimentional PAGE (Hutson & Stocco, 1989). This suggests a possible role of macrophages in the regulation of Leydig cell function in addition to their phagocytic activity. This is supported by studies which have demonstrated that factors secreted by macrophages including arachidonic acid metabolites (Didolkar & Sundaram, 1987; Majercik & Puett, 1991), IL-1 (Calkins *et al.*, 1988; Verhoeven *et al.*, 1988; Béllvé & Zheng, 1989; Fauser *et al.*, 1989; Lin *et al.*, 1991), TNF-α (Matthews, 1981; Calkins *et al.*, 1990), and TGF-ß (Bellvé & Zheng, 1989) can modulate Leydig cell function.

1.4.3.3. Leydig cell-Sertoli cell interactions

The ability of androgens produced by Leydig cells to maintain the process of spermatogenesis led to the investigation of the action(s) of androgen on Sertoli cells which are thought to mediate the effects of androgens on germ cells. Although Sertoli cells have been shown to contain and express the androgen receptor (Tindall *et al.*, 1977; Sar *et al.*, 1990) which is influenced by

hormones, sexual maturation, and the cycle of the seminiferous epithelium (Nakhla *et al.*, 1984; Isamaa *et al.*, 1985; Verhoeven & Cailleau, 1988; Blok *et al.*, 1989), the direct effect of androgens on Sertoli cells *in vitro* was found to be negligible (Louis & Fritz 1979). Peritubular cells were found to be involved in the actions of androgen on Sertoli cells, suggesting that the effects of androgens on Sertoli cell function are mediated by factor(s) secreted by peritubular cells (Skinner & Fritz, 1985b). However, direct effects of androgens on Sertoli cells exist; testosterone was found to cause changes in protein profiles in cultured Sertoli cells which are different depending on age and hormonal condition (Roberts & Griswold, 1989). Also, testosterone and dihydrotestosterone were found to cause a decrease in the levels of plasminogen activator activity secreted by Sertoli cell cultures (Ailenberg *et al.*, 1990).

In addition to androgens, Leydig cells can potentially influence Sertoli cells through nonsteroidal factors. A number of peptides and proteins that can have a regulatory role on Sertoli cells and the seminiferous tubule, have been shown to be produced or localized in Leydig cells. Those factors include renin (Parmentier *et al.*, 1983; Pandey & Inagami, 1986), opioids (Fabbri, 1990) and pro-opiomelacortin (POMC) and related peptides (Margioris *et al.*, 1983; Pintar *et al.*, 1984) including ß-endorphin, α-MSH, and ACTH (Margioris *et al.*, 1983; Valenca *et al.*, 1986). The POMC-derived peptides have been reported to be involved in the regulation of Sertoli cell function (Fabbri *et al.*, 1988; Lebouille *et al.*, 1986). Perfusion of the testis with these peptides revealed

that ACTH, but not α-MSH or ß-endorphin can influence androgen production by Leydig cells (Juniewiez *et al.*, 1988).

The ability of Sertoli cells and the seminiferous tubules to affect Leydig cell function was based on observations that Leydig cell morphology was altered by tubules with abnormal function and spermatogenesis (Rich & de Kretser, 1977; Risbridger *et al.*, 1981). In addition to studies with damaged or abnormal testis, examination of normal testis has revealed that Leydig cell morphology changes with the seminiferous cycle specially at stages VII and VIII (Bergh, 1982, 1983). The morphological observations have been extended by many workers using Leydig cell-Sertoli cell/or seminiferous tubule cocultures. Sertoli cells were found to generally increase basal and hormone-stimulated Leydig cell functions, and FSH was found to indirectly, through the Sertoli cell, stimulate Leydig cell steroidogenesis and cause changes in Leydig cell morphology (Benahmed *et al.*, 1985; Kerr & Sharpe, 1985; Perrard-Sapori *et al.*, 1986). Seminiferous tubules in coculture, particularly at stages VI and VII, were also found to influence the Leydig cell function (Parvinen *et al.*, 1984; Vihko & Huhtaniemi, 1989). Studies in which condition culture medium from Sertoli cells was used were questionable, some of them showed a stimulatory effect (Papadopoulos *et al.*, 1987; Verhoeven & Cailleau, 1986, 1987) while an inhibitory effect has been demontrated by others (Syed *et al.*, 1985; Benahmed *et al.*, 1986). The strongest evidence supporting the regulation of Leydig cell function by Sertoli cells came from studies by Sharpe & Cooper (1984) and subsequently by Risbridger *et al.* (1987). Those studies demonstrated the

presence of a factor in the testicular interstitial fluid (IF) which stimulates basal testosterone production and enhances the responsiveness to hCG. The effect of this factor was found to be more pronouced if IF from cryptorchid testis is used.

Several Sertoli cell-secretory products have been identified that can potentially mediate regulatory interactions between Sertoli cells and Leydig cells. One of the first paracrine factors considered was oestrogen. Sertoli cells are the major sourse of oestrogens before puberty (Dorrington & Armstrong, 1975) and oestrogens, mainly oestradiol, exerts inhibitory effects on androgen production (see section 1.5.3.2. for Refs). However, since before puberty Leydig cells lack oestrogen receptors (Abney & Melner, 1979), the physiological significance of the regulation of Leydig cell function by Sertoli cell-derived oestrogen is questionable.

Another factor postulated to be produced by Sertoli cells that can act on Leydig cells is an LHRH-like substance (see Sharpe 1984, 1986 for reviews). The indirect evidence for LHRH being a paracrine factor was based on studies using hypophysectomized rats. In those studies, treatment with LHRH and its agonists caused a decrease in LH receptors and steroidogenesis (Arimura *et al.*, 1979; Bambino *et al.*, 1980; Clayton *et al.*, 1980; Sharpe, 1980). LHRH receptors have been detected in rat Leydig cells (Bourne *et al.*, 1980; Clayton *et al.*, 1980; Kerr & Sharpe, 1985) and LHRH-like activity was detected in the interstitial fluid of rats treated with hCG (Sharpe & Fraser, 1980) and was found to be secreted by Sertoli cells (Sharpe *et al.*, 1991). LHRH and its

agonists were found to have a biphasic effect on Leydig cells. Acute exposure (up to 24h) stimulates basal and LH-stimulated testosterone production (Hunter *et al.*, 1982; Sharpe & Cooper, 1982; Browning *et al.*, 1983; Sullivan & Cooke, 1983; Sharpe & Harmer, 1983; Molcho *et al.*, 1984; Sharpe & Cooper, 1987), whereas chronic exposure (24 to 72h) decreases the response to hCG stimulation (Hsueh *et al.*, 1981; Cao *et al.*, 1981; Browning *et al.*, 1983; Hsueh *et al.*, 1983; Sharpe & Cooper, 1987). The Leydig cell responsiveness to an LHRH agonist was found to be negatively regulated by LH (Sharpe & Fraser, 1983). The direct acute stimulatory effect of LHRH on Leydig cell steroidogenesis was found to be associated with an increase in PI turnover (Molcho *et al.*, 1984) and to involve arachidonic acid metabolites other than cyclo-oxygenase or lipoxygenase products (Didokar & Sundaram, 1989). For more details about the mechanism of the direct action of LHRH on the testis see Cooke and Sullivan (1985). The chronic inhibitory effect of LHRH on testicular androgen production was found to occur at sites distal to the formation of cAMP (Bourne *et al.*, 1982) and pregnenolone and was suggested to be due to a decrease in the activity of the enzyme 17α-hydroxylase and C_{17-20} lyase (Hsueh *et al.*, 1983). However, although there is a strong evidence which support the concept of a local testicular control by LHRH-like peptide, it cannot be considered as a universal paracrine factor, since no LHRH effect, LHRH-like activity and receptor have been detected in human (Clayton & Huhtaniemi, 1982), Monkey (Mann *et al.*, 1989) and mouse (Wang *et al.*, 1983). In addition to these factors, Sertoli cells produce a number of growth factors

that potentially mediate Sertoli cell-Leydig cell interactions. IGF-I is produced by Sertoli cells (Palmero *et al.*, 1980; Benahmed *et al.*, 1987; Chatelain *et al.*, 1987; Smith *et al.*, 1987; Naville *et al.*, 1990) and can act on Leydig cells to regulate steroidogenesis (Lin *et al.*, 1986, 1987; Nestler, 1989). However, the physiological significance of an IGF-I-mediated regulatory interaction between Sertoli cells and Leydig cells is questioned, since this factor is also produced by Leydig cells (Cailleau *et al.*, 1990; Lin *et al.*, 1990; Naville *el al.*, 1990) and also the Leydig cells are in contact with the liver-derived IGF-I present in the interstitial fluid.

The other regulatory peptide involved in Sertoli/ Leydig cell interaction is inhibin. In addition to its inhibitory effect on FSH secretion, inhibin and its related protein activin can influence Leydig cell steroidogenesis (Hsueh *et al.*, 1987; Lin *et al.*, 1989). Leydig cells have been reported to be involved in the regulation of inhibin production by Sertoli cells (Drummond *et al.*, 1989; Sharpe *et al.*, 1988). However, the ability of Leydig cells to produce inhibin and related peptides (Drummond *et al.*, 1989; Lee *et al.*, 1989; Shaha *et al.*, 1989; Simpson *et al.*, 1991) and the ineffectiveness of inhibin on steroidogenesis in rat tumour (R2C) Leydig cells (Gonzalez-Manchon & Vale, 1989) and in rat Leydig cells (Lin *et al.*, 1989) questions the relevance of Sertoli cell inhibin as a paracrine factor in the regulation of Leydig cell function.

Additional regulatory agents produced by Sertoli cells that can influence Leydig cell function include interleukin-1 (IL-1) (Bellvé & Zheng, 1989, Calkins *et al.*, 1990) and vasopressin-like peptides (Adashi & Hsueh, 1981, 1982; Kasson &

Hsueh, 1986; Sharpe & Cooper, 1987; Tahri-Joutei & Pointis, 1988).

1.4.3.4. Peritubular cell-Sertoli cell interactions

Peritular cell-Sertoli cell interactions are postulated to play an integral role in the maintenance of testis function. Coculture of Sertoli cells and peritubular cells increases the production of androgen binding protein (ABP) by Sertoli cells (Hutson *et al.*, 1987; Verhoeven & Cailleau, 1988); stimulates transferrin production by Sertoli cells (Holmes *et al.*, 1984; Swinnen *et al.*, 1990); and influences the vectorial secretion of proteins by Sertoli cells (Janecki & Steinberg, 1987; Ailenberg *et al.*, 1988). These effects were found to be mediated by a peritubular cell non-mitogenic paracrine factor termed PModS (Skinner *et al.*, 1989). In addition to its stimulatory effect on Sertoli cell functions, PMods exerts an inhibitory effect on Sertoli cell aromatase activity (Verhoeven & Cailleau, 1988).

Additional paracrine factors that have been identified to mediate potential regulatory interactions between peritubular cells and Sertoli cells are growth factors. TGFα has been shown to be synthesized and secreted by both Sertoli cells and peritubular cells (Bellvé & Zheng, 1989, Skinner *et al.*, 1989). However, functional TGFα/EGF receptors are only expressed on peritubular cells and TGFα/EGF stimulates the growth of peritubular cells but not Sertoli cells (Bellvé & Zheng, 1989, Skinner *et al.*, 1989). The few effects of TGFα/EGF on Sertoli cell function which have been reported so far (Morris *et al.*, 1988) were suggested to be due to the impurity of Sertoli cell preparations (Skinner *et al.*, 1989).

1.4.3.5. Sertoli cell-germinal cell interactions

The effects of removal of germ cells on Sertoli cell products *in vivo* (Kasuga *et al.*, 1989; Allenby *et al.*, 1989; Sharpe *et al.*, 1990) support strongly the concept of Sertoli cell-germ cell interactions. This was confirmed by coculture studies. The presence of Sertoli cells in culture with spermatogenic cells has been shown to stimulate germ cell RNA and DNA synthesis (Rivarola *et al.*, 1985).

Several Sertoli cell secretory products have been identified that may mediate regulatory interactions between Sertoli cells and germ cells. IGF-I has been shown to be produced by Sertoli cells (Smith *et al.*, 1987) and may act on meiotic spermatogenic cells via IGF-I receptors. Based on their mitogenic properties on somatic cells, two Sertoli cell-derived growth factors, namely the seminiferous growth factor (SGF) (Feig *et al.*, 1980) and the Sertoli cell-secreted growth factor (SCSGF) (Bellvé & Zheng, 1988), have been speculated to act on germ cells.

The effects of germ cells on Sertoli cells have also been studied. Studies using conditioned medium from germ cell cultures support the regulatory role of germ cells on Sertoli cell function. Germ cell-conditioned medium was found to stimulate the phosphorylation of specific proteins in Sertoli cells (Ireland & Welsh, 1987), increase ABP production and decrease oestradiol synthesis by Sertoli cells (Le Magueresse & Jegou, 1986, 1988ab), increase levels of the inhibin-α subunit mRNA (Pineau *et al.*, 1990), and influence vectorial secretion of proteins by Sertoli cells (Onoda & Djakiew, 1990; Onoda *et al.*, 1991).

Conditioned medium from cultures of pachytene spermatocytes and early stage spermatids were generally found to have most dramatic effects. The factors involved in this process have not yet been characterized.

In addition to the above factors, nerve growth factor (NGF) has also been postulated to be a paracrine factor in the seminiferous tubule (Bellvé & Zheng, 1989; Persson *et al.*, 1990). ß-NGF gene expression and NGF immunoreactivity have been identified in spermatocytes and early spermatids (Ayer-LeLievre *et al.*, 1988). NGF receptor gene expression has recently been identified in Sertoli cells to be under androgen regulation (Persson *et al.*, 1990).

1.4.3.6. Lymphocyte-Leydig and Sertoli cell interactions

The interaction between Leydig cells and lymphocytes is supported by studies in which lymphocyte products have been shown to influence Leydig cell function. Interleukin-2 (IL-2), which is produced by lymphocytes in response to IL-1 (Dinarello, 1984), was found to be a potent inhibitor of Leydig cell steroidogenesis (Guo *et al.*, 1990). In addition, LHRH might be another factor mediating the interaction between the two types of cells, since LHRH mRNA (Azad *et al.*, 1991) and immunoactive and bioactive LHRH (Emanuele *et al.*, 1990) have been detected in rat spleen lymphocytes. A possible interaction between lymphocytes and Sertoli cells has also been demonstrated; transferrin production was found to be regulated by IL-2 and this was found to be dependent on the stage of spermatogenesis (Boockfor & Schwarz, 1991).

1.4.3.7. Mast cell-Leydig cell interactions

Mast cells are frequently encountered in rat testes during recovery from impairment of testicular function (e.g. after treatment with ethane-1,2-dimethanesulphonate (EDS)) in the adult rat and after oestrogen treatment in the neonate (Gaytan *et al.*, 1990). On the other hand, histamine which is produced by mast cells was found to enhance the stimulatory effect of hCG on Leydig cell steroidogenesis in the Golden hamster (Mayerhofer *et al.*, 1989).

1.4.3.8. Autocrine regulation of Leydig cell function by corticotrophin releasing hormone (CRF)

CRF was found to be produced by cultured rat Leydig cells in response to LH/hCG (Fabbri *et al.*, 1990) and to exert negative autocrine modulation on the stimulatory action of LH/hCG on Leydig cell steroidogenesis (Ulisse *et al.*, 1989, Fabbri *et al.*, 1990). The effect of CRF on Leydig cells was found to involve protein kinase-C (PKC) (Ulisse *et al.*, 1990) and to be mediated by endogenous opioids (Eskeland *et al.*, 1989).

1.5. THE MECHANISM OF ACTION OF LUTEINIZING HORMONE

1.5.1. Luteinizing hormone receptor

The LH receptor is a cell surface receptor present on testicular Leydig cells and ovarian mature granulosa cells and theca as well as luteal cells. In both males and females, the LH/CG receptor recognizes the pituitary hormone LH and its placental analogue hCG. Testicular LH receptors increase gradually with advancing age and testicular growth (Thanki & Steinberger, 1976; Ketelslegers *et al.*, 1978; Clausen *et al.*, 1981; Huhtaniemi *et al.* 1982; Bortolussi *et al.*, 1990). The number of LH receptors per Leydig cell is about 1,500 in the human, about 20,000 in the rat, and 60,000 in the pig. A similar species specificity to that in the ovary is seen with testicular LH receptors; the human receptors bind effectively only primate LH preparations (Huhtaniemi, 1983). This species specificity was also found when human LH receptor was expressed in the human foetal kidney cell line (293 cells), the rat LH receptors recognize LH and hCG from rat, human, equine, and ovine species whereas the human receptor only recognizes human gonadotrophins (Jia *et al.*, 1991).

Under the normal conditions, only a small fraction of LH receptors are occupied in order to evoke maximum testosterone synthesis (Catt & Dufau, 1973). The presence of excess of receptors above the number required to mediate acute and maximal steroid response to LH has several implications. One is that full LH responsiveness of adenylate cyclase system could recover more rapidly than testicular LH receptors after LH-induced loss of LH

receptors. Upon binding LH or hCG, LH receptor activates a Gs protein, thereby stimulating adenylate cyclase activity and the subsequent cAMP generation results in increased steroid hormone synthesis and secretion (see Hunziecker-Dunn & Birnbaumer (1985) for a review).

1.5.1.1. Structure of LH receptor

Structural studies on LH receptors have been hampered by the low abundance of this receptor. Nevertheless, sufficient progress has been made in this area. The LH/hCG receptor was found to be a single transmembrane protein containing a large extracellular domain and a smaller intracellular domain (see Ascoli & Segaloff (1989) for a review). Recently, two independent groups have combined established techniques for protein purification with molecular biological approaches to clone and sequence the LH/CG receptor's complementary DNA (cDNA) (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989). These studies provide evidence that the LH receptor is a unique member of the G-protein-coupled receptor class. Transfection studies showed that the cloned receptor is capable of binding hormone and activating adenylate cyclase (McFarland *et al.*, 1989). The primary sequence of the rat and porcine receptor reveals six potential glycosylation sites (McFarland *et al.*, 1989; Segaloff *et al.*, 1990). The porcine and rodent receptors have 87% amino acid identity (Segaloff *et al.*, 1990), and the human LH receptor cDNA displays 89% and 82% homology at the nucleotide level with its porcine and rat counterparts respectively (Jia *et al.*, 1991). Hydropathy analysis of this portion of the molecule suggests the characteristic seven transmembrane domains. The

postulated topology of the LH receptor in the plasma membrane is shown in Figure 1.7).

Unlike the numerous other known G-protein-coupled receptors, these LH receptors have a large (i.e. 65 kDa) extracellular domain which has been demonstrated to be important for LH/hCG binding; cells transfected with a cDNA encoding LH receptor representing only the extracellular domain were found to produce a protein capable of binding hCG with the same high affinity as the full-length receptor (Xie *et al.*, 1990). Gitelman (1990) postulated three possible role for this region: 1) it may function alone to bind ligand, thereby activating the receptor for interaction with G proteins; 2) it may contain only a portion of the ligand binding site, with further interaction between ligand and transmembrane domains essential in ligand binding and receptor activation; or 3) it may function merely to direct ligand binding to the transmembrane domains, but by itself does not form part of the ligand binding site.

In ß-adrenergic, rhodopsin, and muscarinic acetylcholine receptors, an extended third cytoplasmic loop seems to be required for specific interaction with the G protein (see Johnson & Dhanasekaran, 1989 for a review). However, the LH/hCG receptor does not appear to have an extended third cytoplasmic loop, and there is no sequence homology with the ß-adrenergic receptor's G-protein-coupling domain.

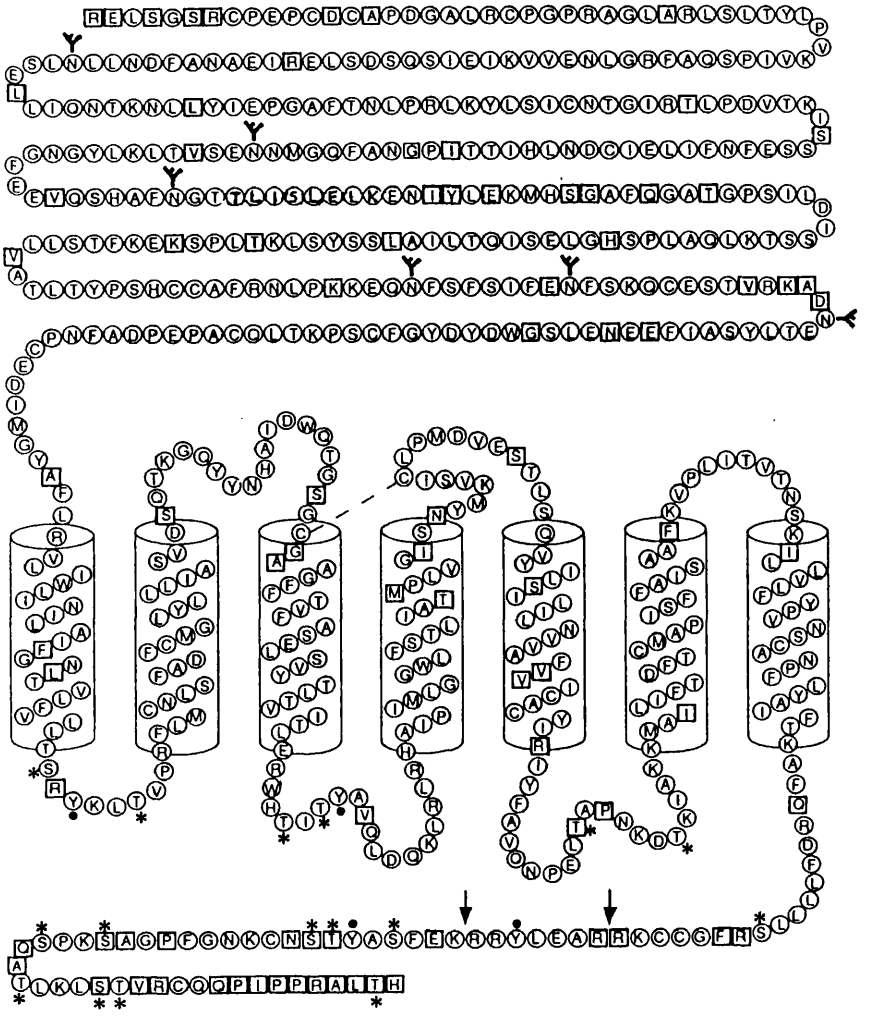


Figure 1.7. Postulated topology of the LH receptor in the plasma membrane. Amino acids that are identical between the rat luteal and porcine testicular LH receptors are enclosed in circles. Those enclosed in squares are unique to the rat luteal receptor. Amino acids in barrels correspond to the putative transmembrane regions, those amino acids above the barrels being extracellular, those below the barrels being intracellular. Potential sites for N-linked glycosylation in the extracellular region are denoted by (Y). A potential disulfide bond between the first and second extracellular loop regions is denoted by the dashed line. Potential intracellular sites for phosphorylation are denoted by asterisks (serine and threonines) or solid circles (tyrosines). The two arrows in the cytoplasmic tail point to two clusters of basic amino acids which might represent potential tryptic cleavage sites (Segaloff *et al.*, 1990).

LH receptors are evolutionarily related to FSH and TSH receptors, which are all characterized by a large extracellular amino-terminal region (Parmentier *et al.*, 1989; Sprengel *et al.*, 1990). A region spanning the second extracellular and third transmembrane domains is highly conserved among the human LH, FSH, and TSH receptors (Jia *et al.*, 1991).

Multiple mRMA species can be identified in gonadal tissues by probes specific for the LH receptor (Loosfelt *et al.*, 1989; Tsai-Morris *et al.*, 1990). A species (rat/ mouse/ human) difference with respect to the relative abundance of gonadal LH receptor transcripts was found although the cell surface receptor expressed is identical. The truncated receptors, lacking the transmembrane domains which may be secreted into the interstitium and blood, were suggested to serve as a binding protein to control gonadotrophin access to the receptor or as a competitive inhibitor (Gitelman, 1990; Segaloff, 1990). Loosfelt *et al.* (1989) reported three short forms of the porcine LH receptor that lack the transmembrane domains which constitute about 40% of LH receptor mRNA.

1.5.2. LH receptor transducing systems

The actions of LH in the Leydig cell are mediated through high affinity cell-membrane receptors. The adenylate cyclase system is generally accepted as the main second messenger for LH (Cooke *et al.*., 1976; Themmen *et al.*, 1986). In addition, other second messenger systems have also been implicated in the action of this gonadotrophin (Figure 1.8) (Rommerts & Cooke, 1988; Cooke, 1990).

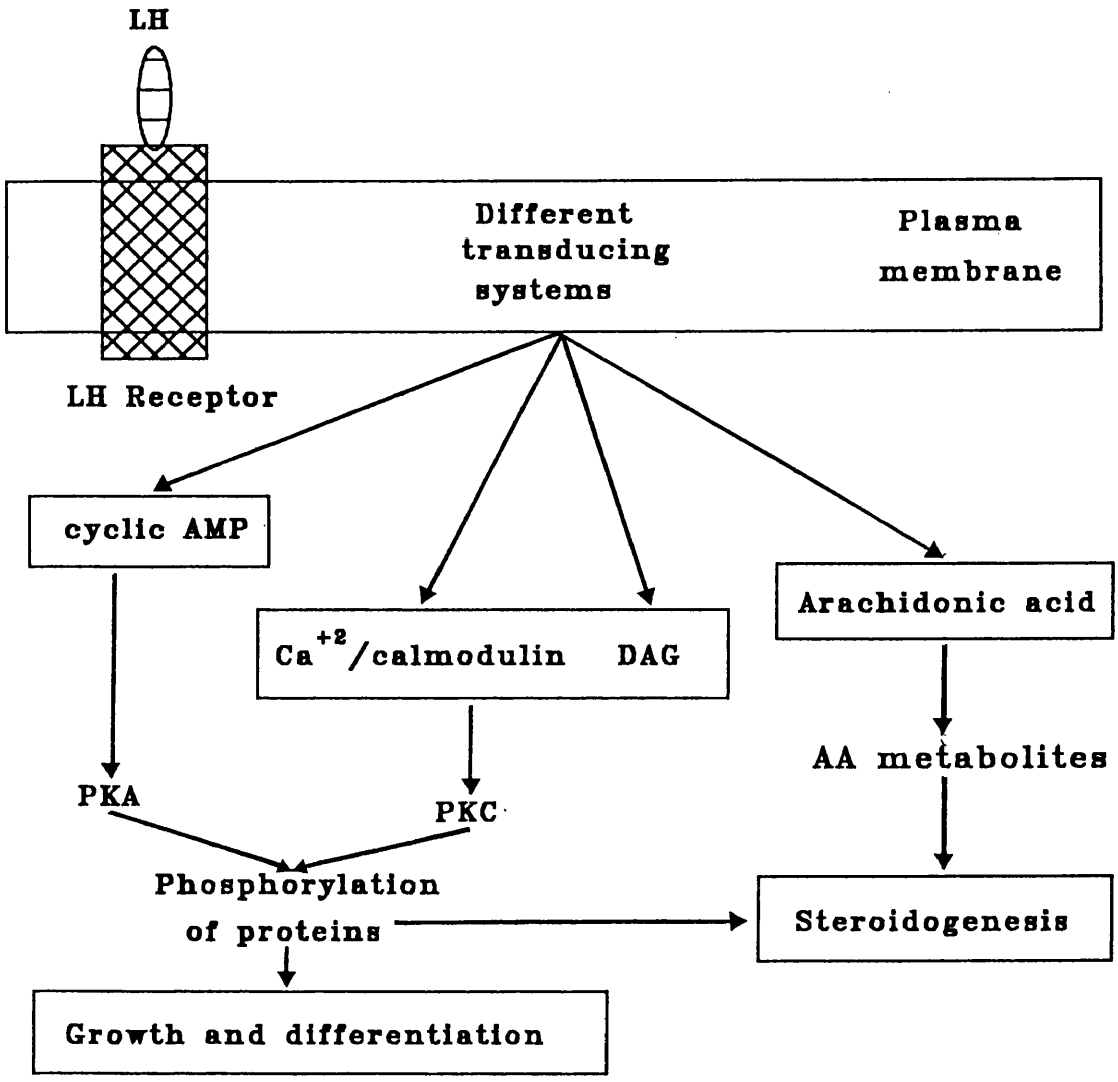


Figure 1.8. Tansducing systems involved in the action of LH/hCG on Leydig cells. LH interacts with its specific receptor in the plasma membrane of the Leydig cell which results in the activation of several transducing systems (AC, PLA_2, PLC, and/or PLD) and the formation of several second messengers (cAMP, Ca^{+2}, DAG, and AA and its metabolites). Protein kinases (A, C, and calmodulin-dependent) are activated resulting in the phosphorylation of specific proteins which leads to the synthesis and/or activation of specific proteins which are involved in the regulation of Leydig cell growth and/or steroidogenesis (Rommerts & Cooke, 1988).

1.5.2.1. Transducing system involving cyclic AMP

Adenylate cyclase (AC) is connected to the receptor/hormone complex by G proteins. The receptors which are coupled to the AC system can be divided into two sub-types: the receptors which interact with G_i inhibit adenylate cyclase (e.g. α_2-type catecholamines, dopamine (D_2 receptor), muscarinic- type acetylcholine, somatostatin, opioids peptides and adenosine (A_1 receptor)) and those which interact with G_s stimulate adenylate cyclase (e.g. ß-adrenergic agonist, LH, FSH, TSH, ACTH, glucagon, and adenosine (A_2 receptor) (Gilman, 1984; Birnbaumer *et al.*, 1985).

1.5.2.1.1. GTP Binding proteins

The G proteins that have been identified so far share a high degree of structural homology. They are all heterotrimeric, comprising of α, ß, and gamma subunits. The specificity in terms of receptor and effector interaction seems to be determined by the α subunit. G proteins are implicated in the transduction of multiple signals including activation and inhibition of AC (Northup *et al.*, 1980; Bokoch *et al.*, 1983), modulation of phospholipase-C (PL-C) (Ohta *et al.*, 1985), and phospholipase-A2 (PLA_2) (Nakamura & Ui, 1985), and gating of Ca^{2+} (Holz *et al.*, 1986) and K^+ channels (VanDongen *et al.*, 1988).

It has now become clear that a single effector system can be regulated by more than one G protein and that a single G protein may regulate more than one effector. For example, the three Gi proteins stimulate the same inwardly K^+ channel with similar potency, also G_s stimulates with similar potency, both

AC and a voltage-gated Ca^{2+} channel (Yatani *et al.*, 1987, 1988).

The concept of a single G protein interacting with various effectors was discussed by Rodbell (1985) who suggested that the activated α-subunit of G proteins released after hormone-receptor interaction can be covalently modified (phosphorylation, sulphation, methylation) and then the modified α-subunit can function as a programmable messenger.

Because of their G protein specificity, and because of the effect they have on G protein function, cholera toxin (CTX) and pertussis toxin (PTX) are powerful tools for investigating possible involvement of a G protein in a cellular response. In contrast to CTX, PTX ADP-ribosylates a much wider spectrum of G proteins. As defined by their functions, these include Gi (the mediator of AC inhibition), at least one form of Gp (the activator(s) of membrane phospholipase activity C and A2 types), of Gk (the stimulator of at least two classes of K^+ channels) (see Birnbaumer *et al.*, 1990 for Refs).

In the Leydig cell, stimulation of AC by LH is mediated by coupling of the LH receptor to AC via a Gs protein (Dix *et al.*, 1982; Cooke *et al.*, 1986). Although the involvement of Gi in the control of AC by LH has not yet been demonstrated, there is strong evidence that Gi is present in rat Leydig cells (Adashi *et al.*, 1984; Platts *et al.*, 1988).

1.5.2.1.2. Adenylate cyclase and cAMP-dependent protein kinases

Adenylate cyclase (AC) is a transmembrane glycoprotein with a molecular mass of approximately 120-150 kDa which catalyses the production of cAMP from MgATP or MnATP substrates (Schlegel *et al.*, 1979). There is both genetic

(Livingstone *et al.*, 1984) and biochemical (Mollner & Pfeuffer, 1988) evidence indicating that the enzyme is expressed as different subtypes. Testicular adenylate cyclase was characterized by Jahnsen *et al.* (1980). Based on cloning of the AC, the topography of this enzyme was determined and was found to belong to the general family of transporter or channel proteins (see Krupinski *et al.*, 1989 for a review).

The diterpene, forskolin, can bind to and activate adenylate cyclase directly without G_s (Seamon & Daly, 1981; Seamon *et al.*, 1984; Lefèvre *et al.*, 1985). In mammalian and other eucariotic cells, the product of adenylate cyclase, cAMP, activates cAMP-dependent protein kinase (PK-A), which like other protein kinases, phosphorylates cellular proteins and thereby modifies their biological functions. In its inactive form, PKA is a heterotetramer of two identical regulatory subunits and two catalytic subunits. There are two types of PK-A, type I and type II (Taylor *et al.*, 1986).

Both kinases are activated by reversibly binding of cAMP molecules to the regulatory subunits, resulting in a dissociation of the holoenzyme as indicated in the following scheme:

$$R_2C_2 \text{ (inactive)} + 4 \text{ cAMP} \longleftrightarrow R_2\text{-}(cAMP)_4 + 2 \text{ C (active)}$$

When cAMP is removed, the catalytic subunits are inactivated by reassociation with the regulatory dimer. Free cAMP, but not cAMP-bound to the regulatory unit, is susceptible to hydrolysis to AMP by the cAMP-phosphodiesterase.

The rat Leydig cell has been shown to contain two forms of cAMP-dependent protein kinase (PKAI and PKAII) (Podesta *et al.*, 1978; Moger, 1991). Both PKA

isoenzymes are also present in mouse Leydig cells although the type I kinase predominates as demonstrated using cAMP analogues selective for either of the two types of PK-A (Hipkin & Moger, 1991).

During incubation of Leydig cells with gonadotrophin, total protein kinase activity was found to be stimulated in a dose-dependent manner with the formation of a single catalytic subunit. However, there is evidence for a differential activation of cAMP-dependent protein kinase during gonadotrophin action; PKAI was found to be activated with lower steroidogenic doses of hCG (1 to 20 pM) whereas PKAII is only activated with the highest hormone concentration (20 pM) (Podesta *et al.*, 1978). This is supported by a study on rat Leydig cells by Moger (1991) in which synergistic increases in androgen production by rat Leydig cells occurred when they were incubated *in vitro* with cAMP analogue pair that selectively activate PKA type I or II. Synergistic increases in androgen production were also observed when either a type I selective analogue (8-aminohexylamino-cAMP or a type II selective analogue (8-thiomethyl-cAMP) is paired with added cAMP. However, when these analogues were paired with low concentrations of LH or forskolin, a synergistic increase in steroidogenesis occured only with the type I selective analogue. Based on these findings, it was suggested that PKAI is compartmentalized in Leydig cells so that it has preferential access to endogenously produced cAMP. However, in MA10 cells, RIIß mRNA but not the mRNA of the others subunits, was found to increase in response to LH/hCG, CTX, forskolin, and 8-bromo-cAMP suggesting that the gene coding for RIIß is positively regulated

by cAMP (Frϕysa *et al.*, 1988).

The role of the cAMP as the second messenger in LH-induced Leydig cell steroidogenesis has been challenged (Themmen *et al.*, 1985; Cooke, 1990). However, this has been based on the inability to detect increased cAMP concentrations in response to low concentrations of LH which are effective in increasing androgen secretion in normal (Rommerts *et al.*, 1972; Dufau *et al.*, 1975; Mendelson *et al.*, 1975; Conn *et al.*, 1977; Sullivan & Cooke, 1986; Choi & Cooke, 1990) and tumour Leydig cells (Peirera *et al.*, 1987). The ability of the inactive cAMP analogue, Rp-cAMP, to inhibit the steroidogenic response to levels of LH which do not initiate any discernible increase in cAMP levels, does however, strongly suggest that the cAMP cascade is involved (Peirera *et al.*, 1987).

1.5.2.2. Transducing system involving inositol phospholipid metabolism

Many lipids or lipid-derived products generated by phospholipases namely phospholipase-A2 (PLA2), -C (PLC) and -D (PLD) acting on phospholipids in membranes are implicated as mediators and second messengers in signal transduction (see Dennis *et al.*, 1991 for a review).

A wide variety of extracellular messengers have been shown to induce turnover of membrane inositol phospholipids in different cell types (see Michell, 1975,1979 for reviews). The agonists which stimulate the release of phosphatidylinositol (PI) initiate a signal cascade resulting in the mobilization of calcium and the activation of protein kinase-C (PKC) (Berridge, 1984). Stimulation of this pathway also leads to the release of arachidonic acid

(Nishizuka, 1984). The signal transducing system involving PI has been a subject of many reviews (Berridge, 1984; Nishizuka, 1984a,b, Majerus *et al.*, 1985; Kikkawa *et al.*, 1986; Nishizuka, 1986; Berridge, 1987; Cockroft, 1987).

1.5.2.2.1. The phosphoinositide cycle

Although there is strong evidence supporting the involvement of PI in the regulation of steroidogenesis in the Leydig cells, its involvement in LH action is still controversial. LH has been shown to increase phosphoinositide metabolism in rat Leydig cells (Lewitt *et al.*, 1982; Malcho *et al.*, 1984). However, Nielsen *et al.* (1989) found that, in rat Leydig cell preparations, PLC can be stimulated with arginine vasopressin (AVP) and bradykinin, but not by LH. Similarly, MA10 cells which also express a hormone-sensitive inositol phosphate/ diacylglycerol (DAG) pathway can be stimulated with AVP, but not by mEGF or LH/hCG (Ascoli *et al.*, 1989).

After phosphatidylinositol bis-4,5-phosphate ($PIP_{2)}$ hydrolysis, the hydrophilic second messenger, inositol trisphosphate (IP_3) is released into the cytosol and the hydrophobic DAG is left within the membrane (Berridge, 1987). The primary function of IP_3 is to mobilize calcium from intracellular stores (see Berridge, 1984, 1987 for reviews). IP_3 acts through a specific receptor located in endoplasmic reticulum (ER) (Berridge, 1987). DAG can activate protein kinase-C (PKC) (See Takai *et al.*, 1984 for a review) by increasing the affinity of PKC for Ca^{2+} resulting in its full activation at physiological Ca^{2+} concentrations (Nishizuka, 1984b).

1.5.2.2.2. The role of Ca^{2+}

Like cAMP, calcium may be involved in many aspects of cell regulation (See Huggins & England, 1985 for a review). The role of calcium as a second messenger was reviewed by Rasmussen (1989). Many hormones increase intracellular calcium levels in their physiological action which is abolished when these increases are inhibited, or when the actions of calcium ions are prevented with specific inhibitors. The calcium ions involved in the increase of the intracellular calcium concentration can either originate from outside the cell, or can be liberated from intracellular stores, the ER and the mitochondria, althought the latter source is considered not to be involved in the hormone-stimulated increase in intracellular calcium. Because high intracellular calcium is cytotoxic (Rasmussen & Barrett, 1984), the cell has an elaborate system of pumps that actively decrease the cytoplasmic calcium: Ca^{2+}-ATP-ases on the plasma membrane, mitochondrion and ER and Na^{+}/ Ca^{2+} exchangers in the plasma membrane.

The functional effects of an increase in intracellular calcium are always mediated by calcium-binding proteins. There are two classes of these proteins: 1) true calcium receptor proteins such as calmodulin, parvalbumin and troponin C, which undergo a conformational change upon calcium binding, and subsequently interact with enzymes dependent on these proteins for their activity, and 2) enzymes directly regulated by calcium, such as calcium-activated protease or PKC. Among the calcium receptor proteins, calmodulin is the most extensively studied (see Means, 1981 for a review). Its molecular

mass is 16 kDa and it has four sites for binding calcium. Upon binding of calcium ions, the protein changes its conformation, becomes active, and binds to the calmodulin-target proteins e.g. Ca^{2+}-dependent phosphodiesterases. Calmodulin was found to be involved in the transport of cholesterol to the mitochondria in the Leydig cells, a process that is stimulated by LH and cAMP (Hall *et al.*, 1981). This is supported by a study by Papadopoulos *et al.* (1990) in which a highly active Ca^{2+}-calmodulin-dependent protein kinase was identified in the cytoskeletons of normal and transformed bovine adrenocortical cells. The activity of this enzyme as well as the increase in steroidogenesis and the transport of cholesterol to the mitochondria in response to ACTH were inhibited by inhibitors of calmodulin (trifluoperazine, pimozide, and W7). In addition Meikle *et al.* (1991) have shown that in mouse Leydig cells, Ca^{2+} is involved in the transport of cholesterol, since testosterone production in response to LH/hCG, but not 22R-hydroxycholesterol, was affected by depletion of extracellular Ca^{2+}.

1.5.2.2.3. The role of protein kinase-C and diacylglycerol

Protein kinase-C has a crucial role in signal transduction for a variety of biologically active substances which activate cellular functions and proliferation (see Nishizuka, 1984 for a review). It is well established that in many cell types PKC is activated by DAG.

There are three isotypes of PKC; Leydig cells, the ovary and the hypothalamus express all the three isotypes (I (δ), II(ß), and III(α) at similar levels and the pituitary express type II and type III, whereas bovine adrenocortical cells,

as well as porcine Sertoli cells express only the type III PKC (Weeler & Veldhuis, 1989; Naor, 1990; Pelosin *et al.*, 1991). PKC was found to be implicated in the regulation of rat Leydig cell steroidogenesis by LH, since phorbol-12-myristate-13-acetate (PMA) causes a decrease in cAMP and testosterone production in response to LH (Moger, 1985; Papadopoulos *et al.*, 1985) but causes a significant increase in testosterone production in the absence of LH (Lin, 1985; Moger, 1985). In addition, LHRH was found to cause translocation of PKC from the cytosol to membrane fractions in Leydig cells (Nikula & Huhtaniemi, 1988). Similarly, it has been found that in calf adrenal glomerulosa cells, ACTH rapidly increases DAG and stimulates the translocation of PKC from the cytosol to the membrane fraction (Cozza *et al.*, 1990). In experiments using phorbol esters or measuring the DAG-stimulated activity of the isolated enzyme, it was shown that PKC is capable of phosphorylating proteins associated with many cell functions including cholesterol side chain cleavage enzyme (Steinschneider *et al.*, 1989).

There is evidence that PKC phosphorylates the catalytic unit of adenylate cyclase in frog erythrocytes (Yoshimasa *et al.*, 1987). PKC probably exerts a similar effect in the Leydig cells, since there is evidence that phorbol ester treatment of Leydig cells can inhibit Gs function (Mukhopadhyay & Schumacher, 1985). In addition, the α-subunits of both G_i and the retinal G protein transducin (TD) in their inactive form were shown to serve as *in vitro* substrates of PK-C (Katada *et al.*, 1985; Sagi-Eisenberg, 1989). The inactivation of G_i by PKC was demonstrated by Platts *et al.* (1988); pretreatment with

12-O-tetradecanoylphorbol-13-acetate (TPA) was found to enhance the subsequent response to cholera toxin. These studies suggest a possible regulatory link between the cAMP and PI-system. Interactions between PKC and PLA_2 has also been reported, PKC was found to stimulate PLA_2 in kidney cells (Parker *et al.*, 1987). In addition the increase in Leydig cell steroidogenesis in response to LHRH and its agonists which involves PKC was found to be mediated via arachidonic acid (Lin, 1985b).

1.5.2.2.4. Role of arachidonic acid and its metabolites

The release of arachidonic acid (AA) from membrane phospholipids is a hormone mediated process involving the activation of PLA_2 and PLD and/or PLC followed by hydrolysis of DAG by 1,2-diglycerol lipase (Mitchell, 1975; Irvin, 1982; Berridge, 1984). PLA_2 and PLC can be coupled to a common membrane receptor by distinct G-proteins and thus can be activated by the same hormone (Lapetina, 1982). The presence of PLD in rat Leydig cells has been recently demonstrated by Vinggaard and Hansen (1991). AA can be metabolized via three independent pathways (Figure 1.9): 1) the cycloxygenase pathway which generate prostaglandins (PGs), prostacyclin (PGI_2) and thromboxanes (Tx), 2) the lipoxygenase pathway which gives rise to hydroxyeicosatetraenoic acids (HETEs) and leukotrienes, and 3) the cytochrome P-450-dependent epoxygenase pathway (Needleman *et al.*, 1986; Axelrod *et al.*, 1988; Smith, 1989). A rapid release of AA has been demonstrated in rat Leydig cells in response to LH (Cooke *et al.*, 1991) and in MA10 cells in response to EGF (Magercik & Puett, 1991). This is supported by the

observation that PLA_2 has a stimulatory effect whereas PLA_2 inhibitors (dexamethasone and quinacrine) exert an inhibitory effect on LH-stimulated Leydig cell steroid production (Abayasekara *et al.*, 1990). Also there is evidence that AA metabolites are involved in the regulation of Leydig cell steroidogenesis by LH (Dix *et al.*, 1984; Didolkar & Sundaram, 1987). Sullivan *et al.* (1988) showed that 5-hydroxyeicosatetraenoic acid (5-HETE), a short-lived metabolite of the 5-lipoxygenase pathway is capable of stimulating Leydig cell steroidogenesis. Other lipoxygenase metabolites including 12-HETE, 15-HETE, and 15-HPETE were also found to have a stimulatory effect on Leydig cell steroidogenesis (Majercik & Puett, 1991). Leukotriene B_4 (LTB_4), which is produced by Leydig cells in response to a calcium ionophore (A23187), but not to LH or LHRH, was found not to be required in the control of steroidogenesis (Sullivan & Cooke, 1985). This is in line with the study by Abayasekara *et al.* (1990) in which no increase in LTB_4 has been detected in the interstitial fluid in response to *in vivo* treatment with hCG at a time when steroidogenesis is increased. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was found to be produced by the testis in rats treated with hCG (Haour *et al.*, 1979) and the stimulation of steroidogenesis and the synthesis of prostaglandins by hCG was found to occur in parallel (Haour *et al.*, 1980). However, the involvement of PGs in the regulation of steroidogenesis by LH was rejected based on studies in which inhibitors of the cylcoxygenase and lipoxygenase pathways were used (Dix *et al.*, 1984; Didolkar & Sundaram, 1987). Furthermore, AA, but not its metabolites, has been found to be involved in the stimulatory effect of LHRH

on Leydig cell steroidogenesis (Lin, 1985b). The direct effect of AA on steroidogenesis in rat Leydig cells was demonstrated by Lopez-Ruiz *et al.* (1992). Some of the effects of AA on Leydig cell steroidogenesis may involve PKC, since the PKC I (gamma) which is present in Leydig cells (Pelosin *et al.*, 1991) was found to be regulated by AA (McPhail, 1984; Dell & Severson, 1989).

1.5.3. Regulation of LH receptor

The maintenance of Leydig cell function and androgen secretion is dependent upon luteinizing hormone, which is released from the pituitary gland in a pulsatile manner and circulates in relatively low concentrations (about 10^{-10}M). Intermittent stimulation by LH is responsible for supporting the differentiated function of the Leydig cell, and induction and/or maintenance of LH receptors and steroidogenic enzymes occurs with near-normal or slightly increased levels of gonadotrophin (Dufau & Catt, 1978; Catt *et al.*, 1979).

1.5.3.1. Desensitization and down regulation

During short term exposure (up to 24h) of Leydig cells *in vitro* to LH/hCG, although there is no net loss of LH receptors (Catt *et al*, 1979), the receptor was found to be in a highly dynamic state being continually internalized into endoplasmic vesicles and recycled back to the cell surface (Cooke *et al.*, 1986).

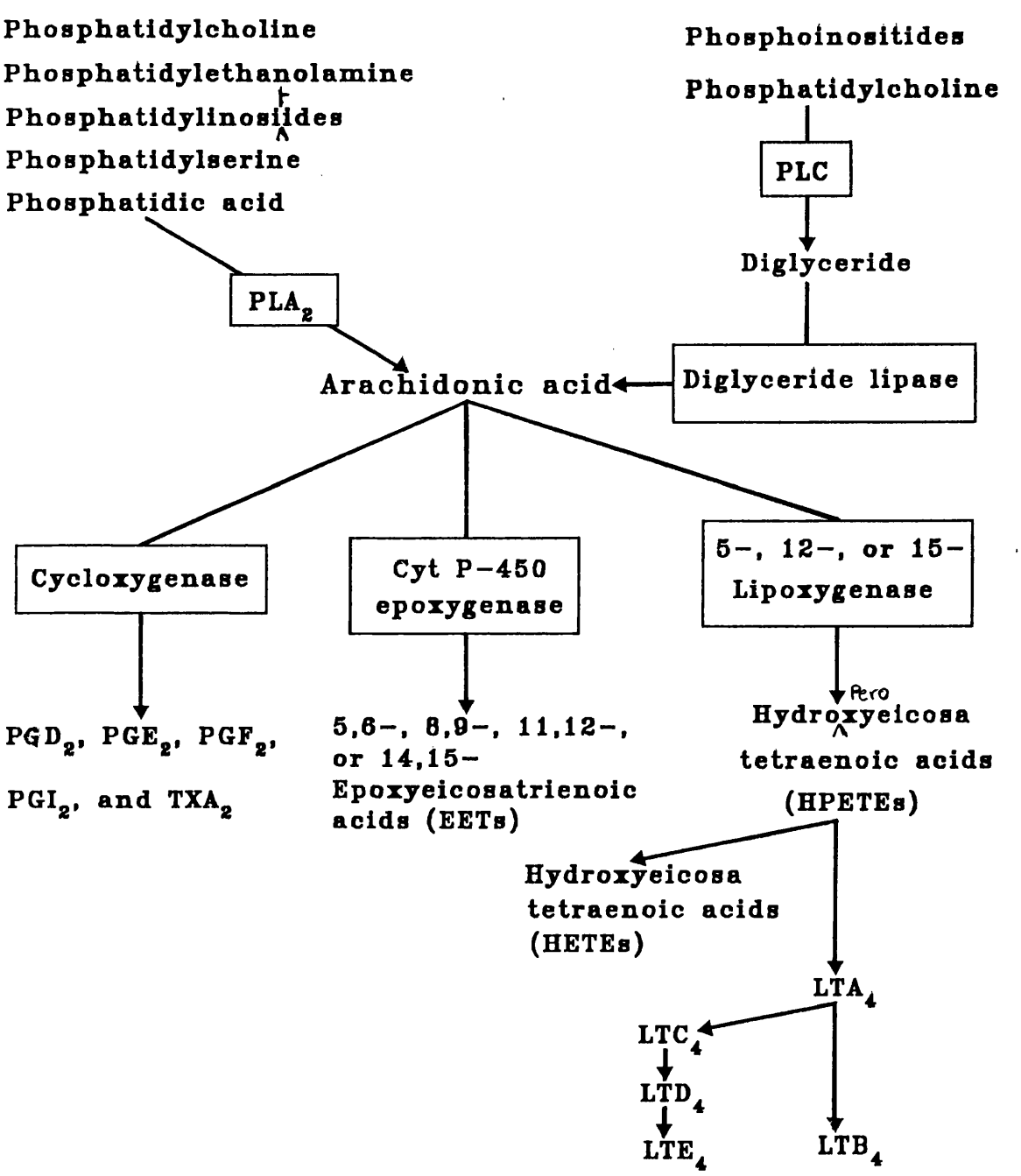


Figure 1.9. Pathways for the generation and metabolism of arachidonic acid. Arachidonic acid (AA) can arise directly from phospholipids through the action of PLA_2 or prior action of PLC, followed by the action of diglyceride lipase. Alternatively, the diglyceride may be phosphorylated to phosphatidic acid by action of diglyceride kinase, and AA then can be released through the action of PLA_2. The release of AA may then be metabolized by lipoxygenase, cycloxygenase, or epoxygenase enzymes to form leukotrienes (LTs), hydroxyecosatetraenoic acids (HETEs), prostaglandins (PGs), thromboxanes (Txs), and epoxides (Axelrod *et al.*, 1988; Smith *et al.*, 1989).

The endocytic pathway was found to have two temperature sensitive steps. At 4°C movement of the hormone/ receptor complex inside the cell does not occur, and at 21°C there is hormone accumulation within the cytoplasm without degradation or release from the cell. At 34°C, internalization, degradation and loss of the degraded hormone fragment from the cell occurs (Habberfield *et al.,* 1986). Continued exposure of the Leydig cells to LH/hCG, however, leads to a desensitization and eventually to down-regulation of LH receptors (see Rommerts & Cooke, 1988 for a review). Example of the down-regulation of LH receptor include the homologous down-regulation which occur after exposure to LH or hCG and the heterologous down-regulation by certain hormones, growth factors, and second messenger analogues (Sharpe, 1976; Cooke & Rommerts, 1988; Segaloff *et al.*, 1990; Lane *et al.*, 1991). LH receptor down-regulation has also been demonstrated following elevated endogenous LH levels by administration of GnRH and its analogues (Catt *et al.*, 1979) and after induction of cryptorchidism in adult rats which results in high circulating levels of LH (de Kretzer *et al.*, 1979). In rats, it can also be caused by a direct effect of GnRH on the testis (Arimura *et al.*, 1979; Hsueh & Erickson, 1979; Bambino *et al.*, 1980).

1.5.3.1.1. Mechanisms involved in the down-regulation of LH receptor

Although LH receptor regulation has been studied extensively, little is known about the mechanisms of regulation. Potential sites for such regulation include alteration in the rate of receptor synthesis, either at the transcriptional or translational level, alteration in receptor stability at the mRNA or protein

level, and alteration in coupling of the receptor to its effector. In MA10 cells, hCG and dcAMP reduces surface LH receptors by increasing the rate of receptor degradation while EGF exerts the same effect by decreasing the rate of receptor synthesis (Lloyd & Ascoli, 1983). Glucocorticoids also have a negative effect on LH receptors (Bambino & Hsueh, 1981).

Several reports have shown that proteolytic cleavage of hormone receptors at the plasma membrane is also involved in down regulation of LH receptors (Hatzfield *et al.*, 1982; Gross *et al.*, 1983; Strulovici & Lefkowitz, 1984; West & Cooke, 1991). The possible involvement of a membrane protease(s) as a modulator of LH receptor function has also been suggested. Kellokumpu and Rajaniemi (1985) have shown that in ovarian membranes, LH receptor in the occupied state is cleaved at two distinct sites by plasma membrane proteinases. Hwang and Menon (1984) also demonstrated a similar process in collagenase-dispersed luteal cells.

The microtubules are also implicated in the process of LH receptor down-regulation, since vinblastine and colchicine which disrupt the microtubule system prevent the loss of LH receptors (Laws *et al.*, 1984).

Although suppression of LH-binding sites may result from ligand-induced receptor internalization, sequestration, and/or phosphorylation, the gonadotrophins also regulate receptor mRNA levels (Pakarinen *et al.*, 1990). The ligand-induced LH receptor down-regulation was found to be preceded by decreased LH/CG receptor mRNA transcripts of 7, 4.2, and 2.5 kb in both ovaries and testes. However, tissue-specific differential regulation of LH

receptor mRNA was demonstrated, the truncated 1.8-kb species lacking a portion of the transmembrane domain is hormonally regulated in the ovaries but not the testes (Hoffman *et al.*, 1991; LaPolt *et al.*, 1991). The LH receptor of MA10 Leydig tumour cells is down-regulated by LH/hCG, mEGF, cAMP analogues and phorbol esters and this was found to be associated with a reduction in LH receptor mRNA, suggesting the involvement of PKA, PKC and probably EGF-dependent protein kinase in this process (Wang *et al.*, 1991).

1.5.3.1.2. Mechanisms involved in the desensitization of LH/hCG receptor

The responsiveness of the target cell to LH or hCG might also be modulated by alterations in the "functional" activity of the receptor which can occur independently (or in addition to) changes in receptor numbers. A classical example of this phenomenon is that of desensitization, in which the ability of the hormone/receptor complex to activate adenylate cyclase is attenuated (Dix *et al.*, 1982). This can involve homologous as well as heterologous desensitization (Segaloff *et al.*, 1990).

By analogy with the ß-adrenergic receptor (Hausdorff *et al.*, 1990; Lefkowitz *et al.*, 1990), it has been suggested that the desensitization of LH receptor occurs as a result of receptor phosphorylation. Minegishi *et al.* (1987) have reported that the purified receptor from rat testes can be phosphorylated *in vitro* by the catalytic subunit of the cAMP-dependent protein kinase (PKA), but this did not affect the binding affinity for hCG or the maximal amount of hCG bound, suggesting that PKA is not involved in this process. Since addition

of phorbol esters (Rebois & Patel, 1985), calcium ionophores (Pereira *et al.*, 1988a), or epidermal growth factor (Pereira *et al.*, 1988b) also uncouples the LH/hCG receptor from adenylate cyclase, protein kinases such as PKC, or other Ca^{+2}-dependent kinases as well as epidermal growth factor-dependent kinases may be involved in the process of desensitization as a result of receptor phosphorylation.

1.5.3.2. Inhibition of steroidogenesis in LH/hCG desensitized cells: role of testicular oestrogens

In the adult rat the early androgen response to LH or hCG is soon followed by a refractory period in which the testosterone responses to gonadotrophin and exogenous cAMP are markedly impaired for several days after hormone treatment (Cigorraga *et al.*, 1978). The hormone-induced steroidogenesis refractoriness of Leydig cells cannot be entirely explained by the alteration of coupling and eventual receptor loss, since desensitized cells become resistant to the steroidogenic effect of exogenous cAMP and cholera toxin, which indicates a post cAMP block (see Saez *et al.*, 1979 for a review). The nature of the steroidogenic lesions was characterized in the Leydig cells from hCG-treated rats. Although high doses of hCG cause a decrease in the formation of pregnenolone from cholesterol ('early' lesion) (Charreau *et al.*, 1981; Aquilano *et al.*, 1985; Payne *et al.*, 1985), lower doses of hCG do not impair the side-chain cleavage step but still cause a marked fall in testosterone production by inducing a 'late' steroidogenic lesion with reduction of the activities of the microsomal enzymes 17α-hydroxylase/ 17-20 lyase

(Chasalow *et al.*, 1979; Dufau *et al.*, 1979a; Nozu *et al.*, 1981). This defect is attributed to the inhibitory effect of intratesticular oestradiol formation upon testosterone biosynthesis, since a similar lesion can be induced by oestrogen treatment and prevented by the oestrogen antagonist, tamoxifen (Hsueh *et al.*, 1978; Dufau *et al.*, 1979a; Saez *et al.*, 1979; Brinkman *et al.*, 1980; Melner & Abney, 1980; Nozu *et al.*, 1981a,b,c; Keel & Abney, 1982). In addition, the steroidogenic lesion was found to be preceded by activation of aromatase activity with an increased endogenous oestradiol production (Dufau *et al*, 1979b; Forest *et al.*, 1979; Cigorraga *et al.*, 1980; Nozu *et al.*, 1981ab; Aquilano & Dufau, 1983; Tsai-Morris *et al.*, 1985), and oestradiol receptor mediated responses (Brinkmann *et al.*, 1972; Nozu *et al.*, 1981b) such as activation of oestradiol dependent RNA polymerase activity (Nozu *et al.*, 1981b; Aquilano & Dufau, 1983) and synthesis of oestradiol-regulated protein of Mr 27kDa (Nozu *et al.*, 1981c). The direct inhibitory effect of oestradiol on cytochrom $P450_{17\alpha}$ was questioned by Payne and coworkers who attributed the decrease in this enzyme in desensitized Leydig cells to the initial increase in testosterone in response to LH/hCG (Quinn & Payne, 1985; Rani & Payne, 1986; Hales *et al.*, 1987).

In contrast to the rat testis where there is a 'late' steroidogenic lesion after treatment with hCG *in vivo,* desensitization of the steroidogenic response in the mouse testis is associated with decreased pregnenolone synthesis without any change in pregnenolone metabolism to androgens. In murine Leydig tumour cells (M5480A), oestradiol-17ß (Zimniski *et al.*, 1985), hCG, cholera toxin and

8 bromo-cAMP (Segaloff *et al.*, 1981) were found to cause an 'early' lesion in steroidogenesis.

There is a controversy with respect to the depletion of intracellular cholesterol being the cause of the 'early' lesion in steroidogenesis; Quinn *et al.* (1981) and Freeman and Ascoli (1982) support this concept while other studies by Segaloff *et al.* (1981), Charreau *et al.* (1981), Benahmed *et al.*, 1983, and Aquilano *et al.* (1985) failed to confirm those results, suggesting that other mechanisms may be involved in this process. There is evidence that oestrogen treatment results in a marked reduction in peripheral benzodiazepine binding sites in the testis (Gavish *et al.*, 1986) which may contribute to the steroidogenic lesion. This process of desensitization which is attributed to oestrogens does not occur in the foetus or neonate, but is a result of pubertal sexual development (Huhtaniemi *et al.*, 1982; Tsai-Morris *et al.*, 1986; Huhtaniemi & Warren, 1990; Pakarinen *et al.*, 1990). In the rat, the lesion in steroidogenesis coincides with the appearance of oestrogen receptors which are first detectable at 23 days of age, increase dramatically at 7 weeks, and rises only gradually after maturity (Abney & Melner, 1979).

1.6. LEYDIG CELL DEVELOPMENT

After the gonads first form, mesenchyme cells proliferate to yield differentiated interstitial cells, including the steroidogenic Leydig cells. A characteristic feature of the developing mammalian testis is the appearance of the two generations of Leydig cells, i.e. the foetal and adult Leydig cells which exhibit distinct morphological and functional properties (Christensen, 1975; Huhtaniemi *et al.*, 1984; de Kretzer & Kerr, 1988; Bortolussi *et al.*, 1990). In the rat foetal Leydig cells are recognizable at day 10 postnatal and their absolute volume and number per testis increase from 15 to 90 days of age (Mendis-Handagama *et al.*, 1987; Bortolussi *et al.*, 1990). Contrary to the concept of a biphasic pattern of Leydig cell development in which foetal Leydig cells are believed to decrease in number and finally to disappear from the interstitium during a process of regression after birth (Gondos, 1977), more recent reports suggest the presence of 3 consecutive stages: foetal (foetal cells in foetal testis, early juvenile (foetal cells during neonatal-early juvenile life), and juvenile-adult (adult cells before and after puberty) (Kupio *et al.*, 1989a). This pattern is similar to that in the pig (de Kretzer & Kerr, 1988). This latter concept supports the view that the total number of foetal Leydig cells in testes does not change significantly between the end of pregnancy and early days of postnatal life, although the number of cells per unit volume of the testis decreases (Mendis-Handagara *et al.*, 1987; Zirkin & Ewing, 1987; Kerr & Knell, 1988; Kupio *et al.*, 1989a,b). During and after the second postnatal week, foetal Leydig cells show signs of regression (Roosen-Runge & Anderson,

1959; Kuopio *et al.*, 1989a). The foetal Leydig cells which persist after puberty (Kerr & Knell, 1988) appear to be an unimportant minority when compared with the adult-type cells which may be more than 200 to 500 fold higher in number (Mori & Christensen, 1980; Tapanainen *et al.*, 1984; Mendis-Handagama *et al.*, 1987; Kerr & Knell, 1988).

1.6.1. Leydig cell precursors

The origins of Leydig cells in the embryonic testis remain unknown. With respect to the origins of adult Leydig cells, it was suggested that the connective tissue cells which increase in absolute volume with increasing age (Mendis-Handagama *et al.*, 1987) and specifically mesenchymal cells (Kerr & Knell, 1988) could be a source for adult Leydig cell precursors. This was subsequently confirmed by Hardy *et al.* (1989) who demonstrated a precursor-product relationship between mesenchymal and Leydig cells, but no such reciprocal relationship was observed between Leydig cells and macrophages, pericytes, endothelial, or myoid cells. Rapid growth of the mature Leydig cell population was found to be initiated by recruitment of mesenchymal precursor cells, and this occurs preferentially (though probably not exclusively) during days 14-28 postnatal. Thereafter, a relatively high proportion of the newly generated Leydig cells remains in the cell cycle dividing one or two times to complete their final growth phase resulting in a population of 30 to 35 million Leydig cells per testis achieved between 28-56 days postnatal (Mori & Christensen, 1980; Ewing & Zirkin, 1983; Hardy *et al.*, 1989).

1.6.2. Regulation of Leydig cell development

The postnatal growth phase of the foetal Leydig cells was suggested to be regulated by the physiological rise in serum LH during the first week of life, since a direct correlation was found between the increase in serum LH and the number of Leydig cells per testis (Lee *et al.*, 1975; Ketelslegers *et al.*, 1978; Ramaley, 1979). This was subsequently supported by a study by Kuopio *et al.*, 1989b which showed a marked and rapid increase in the number of foetal Leydig cells in newborn rats after exogenous hCG administration. On the other hand, the stimulus for the transformation and/or appearance of the adult generation of Leydig cells remains unknown. It has been assumed that this process is principally dependent upon LH, as judged by the responses to deprivation or stimulation with LH or hCG (Chemes *et al.*, 1976; Christensen & Peacock, 1980). However, no dramatic changes occurred in serum LH levels at this time, which represents the onset of the rise in FSH associated with sexual maturation commencing around 21st to 23rd postnatal day and becoming maximally abundant by day 40 (Lee *et al.*, 1975). The possibility that FSH is the stimulus is supported by the earlier studies of Swerdloff *et al.* (1971) who showed an increased sensitivity of Leydig cells to LH after treatment of immature rats with FSH. More importantly, a study by Kerr & Sharpe (1985) has demonstrated a significant increase in the number of Leydig cells in immature hypophysectomized rats treated with FSH when compared with LH alone. Thereafter, in adulthood, Leydig cells usually do not proliferate (Cameron, 1970; Fouquet & Kann, 1987), the rate of proliferation,

i.e. cell division, is thought to be so slow that not all Leydig cells renew within a lifetime (Cameron, 1970; Teerds *et al.*, 1989d). However, proliferation of Leydig cells occurs in response to non-physiological conditions such as, local implants of testosterone antagonist or an antiandrogen (cyproterone acetate) (Aoki & Fawcett, 1978); administration of prolonged and supra-physioloical levels of exogenous hCG (Schoen, 1964; Aoki & Massa, 1972; Christensen & Peacock, 1980; Teerds *et al.*, 1988, 1989a); cryptorchidism (de Kretzer *et al.*, 1979; Risbridger *et al.*, 1981; Kerr & Donachie, 1986); testicular irradiation (Choen, 1964) and depletion of interstitial cells by treatment with the cytotoxic drug, EDS (Rommerts *et al.*, 1985; Molenaar *et al.*, 1986; Edwards *et al.*, 1989; Teerds *et al.*, 1989b). Leydig cells aquire the ability to proliferate during 2-6 week-period following EDS treatment.

1.6.2.1. Regulation of Leydig cell growth by LH/hCG

The factors regulating Leydig cell proliferation and total number per testis during adulthood are still unknown. LH/ hCG has long been recognized to be the main regulator of Leydig cell proliferation and differentiation. However, only a few studies have quantified the effects of LH on Leydig cell proliferation and total number in adult rats. Treatment with exogenous LH or hCG has been shown to increase ^{3}H-thymidine incorporation by Leydig cell nuclei (Abney & Carswell, 1986; Teerds *et al.*, 1988). Also hypophysectomy results in atrophy and reduction in the number of Leydig cells and treatment with LH prevented Leydig cell atrophy and loss of steroidogenic function (Ewing & Zirkin, 1983). In addition, Leydig cell clusters in the adult testis

undergo a striking increase in volume during chronic stimulation with excess LH or hCG. This change was first noted by Greep *et al.* (1936) and Greep (1937) and was generally assumed to result from increase in the number of Leydig cells and in the size of individual cells (Christensen, 1975). Although there was some evidence that the number of Leydig cells in the rat testis did increase under chronic treatment with hCG (Schoen, 1964), the counting method was subject to criticism (Heller *et al.*, 1971) and therefore could not be conclusive. Further studies have shown that the volume increase in Leydig cell clusters with gonadotrophin treatment is due primarily to an enlargement of the individual cells and that the number of cells remains relatively constant. After chronic hCG treatment (4000 IU two or three times for 6 or 16 weeks), biopsies from human testes showed larger Leydig cell clusters without any significant change in the number (Heller & Leach, 1971). A similar effect was found in beagles (Walkers *et al.*, 1988) with high endogenous LH levels and rats treated with hCG (100IU/kg body weight) for 3 and 6 days (Andreis *et al.*, 1989). However, Christensen & Peacock (1980) demonstrated an increase in the number of Leydig cells as well as an enlargement of the individual cells, when Sprague Dawley rats were treated with 100 IU hCG for up to 5 weeks. Also Teerds *et al.* (1989a) have demonstrated that in the adult cynomolgus monkey, daily hCG treatment for 16 days results in an increase in the number of Leydig cells. In addition LH treatment for 10 days was found to induce hypertrophy and hyperplasia of Leydig cells in hypogonadal (hpg) mice (Scott *et al.*, 1990). It is unclear whether the divergent effects of

LH/hCG on Leydig cell number are the result of species differences, differences between exogenous and endogenous sources of LH, or differences in the doses of LH/hCG administered.

1.6.2.2. Regulation of Leydig cell growth by testicular factors

In addition to LH, other endocrine and/or paracrine factors, both stimulatory and inhibitory, may be involved in the regulation of Leydig cell growth. This is supported by a study by Keeney *et al.* (1990) in which the number of Leydig cells per testis was found to be largely unresponsive to inhibition and subsequent restoration of endogenous LH secretion. Furthermore, several studies have shown that the seminiferous tubules are involved in this process. For example Aoki & Fawcett (1978) have demonstrated that experimental depletion of spermatogenic cells from the seminiferous tubules results in the hyperplasia and hypertrophy of Leydig cells in the vicinity of the affected tubules. It has also been reported that treatment of hypophysectomized immature rats with FSH resulted in an increase in the number of Leydig cells *in vivo* (Kerr & Sharpe, 1985; Teerds *et al.*, 1989c). Recently, it was reported that hyperplasia of Leydig cells also occurs in the testes of men with germ cell tumours. In all the patients, concentrations of plasma hormones were found to be normal and mitotic figures of Leydig cells were observed only in intratubular tissues that were adjacent to the tumour, neighbouring normal tubules showed no evidence of an increase in number. The involvement of paracrine factors in the regulation of Leydig cell growth is also supported by a study by Kerr & Sharpe (1985) and Teerds *et al.* (1989c) which demonstrated an increase in Leydig

cell number in response to FSH. This is probably mediated via paracrine factors secreted by Sertoli cells (de Kretzer, 1982; Sharpe, 1983), since Leydig cells have no FSH receptors (Yoon *et al.*, 1987). These observations are supported by the presence of mitogenic activity in rat testicular interstitial fluid 4 weeks after EDS treatment, a time when cell regeneration occurs (Drummond *et al.*, 1988), and also the fact that Leydig cell regeneration was found to be faster in the cryptorchid testis (O'Leary *et al.*, 1986). These observations were confirmed by the isolation of a Sertoli cell-secreted protein(s) (SCSP) which stimulate DNA synthesis and proliferation of A431 and Swiss 3T3 cell lines (Bellvé & Feig, 1984; Buch *et al.*, 1988; Bellvé & Zheng, 1989; Lamb *et al.*, 1991). Hormes *et al.* (1986) suggested that the mitogenic substance(s) isolated from Sertoli cells may play a role in spermatogenesis and/or the regulation of Leydig cell number. This was confirmed by Ojeifo *et al.* (1990) who demonstrated the secretion of protein(s) (MW ≥ 30kDa) by Sertoli cells, *in vitro*, which stimulate replicative DNA synthesis in purified preparations of Leydig cells from immature and adult rats *in vitro*, and increases the number of immature Leydig cells in culture. However, the *in vivo* effects of FSH treatment on Leydig cells in intact mature rats have not yet been reported. In adult cynomolgus monkeys, FSH had no significant effect on Leydig cell number and testosterone production (Teerds *et al.*, 1989a). In addition FSH was found not to be required for the development of Leydig cells in mature rats after treatment with EDS (Molenaar *et al.*, 1986).

1.6.2.3. The possible mechanisms involved in Leydig cell growth in response to LH/hCG and testicular factors

The mechanism responsible for the growth promoting effect of LH/hCG on Leydig cells remains unclear. Recent studies support a central role of proto-oncogenes in cell proliferation (Slamon *et al.*, 1984; Hunter, 1985; Muller, 1986; Bishop, 1987; Bourne, 1987; Keski-oja & Moses, 1987; Marshall, 1987; Adinolfi, 1988; Fuller, 1988; James *et al.*, 1988; Nowell & Croce, 1988; Rempel & Johnston, 1988; Terrier *et al.*, 1988; Franklyn & Sheppard, 1989; Huet-Hudson *et al.*, 1989; Steel, 1989; Velu *et al.*, 1989; Carbone & Levine, 1990; Lyons *et al.*, 1990; Reure *et al.*, 1990; Velu, 1990). Elevated proto-oncogene expression has been demonstrated in embryonic and neonatal tissues, in cultured cells stimulated with growth factors, in regenerating liver tissue after partial hepatectomy and in a number of malignant tumours (Muller *et al.*, 1982; Kelly *et al.*, 1983; Greenberg & Ziff, 1984; Klein & Klein, 1985; Weinberg, 1985). Elevated expression of the c-myc oncogene is often associated with cell proliferation (Pfeifer-ohlsson *et al.*, 1984; Slamon & Cline, 1984; Marshall, 1987; Rempel & Johnston, 1988; Terrier *et al.*, 1988; Huet-Hudson *et al.*, 1989; Franklyn & Sheppard, 1989). There is evidence that the growth promoting effect of LH/hCG on Leydig cells may be mediated by the transient expression of c-myc proto-oncogene (Lin *et al.*, 1988). Also high levels of a c-myc oncogene product has been detected in human testicular cancer (Sikora *et al.*, 1985; Watson *et al.*, 1986) and in the ovary in response to gonadotrophins (Delidow *et al.*, 1990). In addition to proto-oncogenes, the effect of LH/hCG

could be mediated by growth factors produced locally in the testis. The growth factors which are known as potent mitogens and exist in the testis have been described by Bellvé & Zheng (1989). Those factors include the seminiferous growth factor (SGF), basic fibroblast growth factor (bFGF), IGFI, Sertoli cell-secreted growth factor (SCSGF), TGFα, IL-1α, and ß-NGF. These growth factors have been shown to exist in mammalian testes, based on direct biological assays, biochemical characterization and purification, and/or specific immunological and molecular probes. In addition to these growth factors, other growth factors including EGF and IGFII, appear to reach the gonads via the vascular circulation. The receptors for most of these growth factors function as tyrosine-specific protein kinases, and enhancement of these kinds of stimulatory signals promote cell proliferation in normal and neoplastic tissues (Keski-Oja & Moses, 1987). Leydig cells were found to exhibit differential levels of tyrosine kinase activity, with Leydig cells from population I having higher levels of this enzyme than those from population II (Dangott *et al.*, 1983). In addition to stimulatory growth factors, various factors (e.g. interferons, tumour necrosis factors (TNFs), and TGF-ß) that inhibit cell growth are also secreted by cells and exert paracrine and/or autocrine effects to regulate cell growth (Keski-Oja & Moses, 1987; Marshall, 1987; Wilding *et al.*, 1989; Moses *et al.*, 1990). IGF-I and TGF-α were found to exert a synergistic stimulatory effect on DNA synthesis in Leydig cells from immature rats. On the other hand TGF-ß had a stimulatory effect under basal conditions, but inhibited the cells treated with IGF-I and TGF-α (Khan *et al.*, 1991). The growth promoting

effect of some growth factors may be mediated via cellular oncogenes, since similarities have been found between growth factors or their receptors and the protein products of oncogenes (e.g. c-sis oncogene encodes for the B-chain of platelet-derived growth factor (PDGF) and c-erb oncogene encodes for a protein which is a truncated form of the receptor of EGF) (Sporn & Roberts, 1985; Berridge, 1986; Adinolfi, 1988; Pandiella *et al.*, 1989; Velu *et al.*, 1989; Berridge, 1986; Adinolfi, 1988; Pandiella *et al.*, 1989; Velu *et al.*, 1989; Carbone & Levine, 1990; Travali *et al.*, 1990; Velu, 1990). Also expression of c-myc is activated by a variety of peptide growth factors including PDGF, FGF, and EGF. Stimulation of c-myc expression by most peptide growth factors was found to be primarily at a post transcriptional level due to stabilization of c-myc mRNA (Franklyn & Sheppard, 1989).

The effect of LH/hCG on Leydig cell growth may be mediated through oestradiol, since this steroid is a well known proliferative agent in many endocrine systems including breast cancer cells (Franklyn & Sheppard, 1989), uterus (Huet-Hudson *et al.*, 1989; Franklyn & Sheppard, 1989), and oviduct (Rempel & Johnston, 1988). Furthermore, oestradiol stimulation is always accompanied by accumulation of c-myc oncogene and in some systems, c-fos and ras oncogenes are also induced (Franklyn & Sheppard, 1989). The induction of c-myc by oestradiol was suggested to be mediated by growth factors while that of c-fos is direct, since the effects were quicker on c-fos and the secretion of growth factors such as TGFα and IGFI is enhanced by oestrogen treatment. This observation was supported by the finding that oestrogen

receptor bind to fragments of c-fos (but not myc) genes from several species *in vitro* (Franklyn & Sheppard, 1989).

The effects of trophic hormones and other agents which promote cell growth involves the main transducing systems. Three different mitogenic pathways have been delineated in mammalian cells: (1) the growth factor protein tyrosine kinase pathway, (2) the phosphatidyl-inositol-Ca^{+2}-DAG cascade, (3) the cAMP system which involves PKA activation and phosphorylation of specific substrates (Rosengurt, 1986; Bourne, 1987; Vallar *et al.*, 1987; Dumon *et al.*, 1989; Carbone & Levine, 1990; Reure *et al.*, 1990). Depending on the cell type, two or three of these pathways may be operational. The activation of these cascades was found to induce the expression of the proto-oncogenes c-fos and c-myc (Reure *et al.*, 1990). Any of the genes encoding normal components of these pathways may become mutated and thus result in uncontrolled cell proliferation. Most oncogene mutations promote tumour growth by inducing autonomous activity of proteins, which normally transmit growth signals that are triggered by extracellular factors (Carbone & Levine, 1990). Such mutations have been found in pituitary tumours (mutation of α_s of G_s) (Bourne, 1987; Vallar *et al.*, 1987; Lyons *et al.*, 1990) and in adrenal and ovarian tumours (mutation in α_i of G_{i2}) (Lyons *et al.*, 1990).

1.7. DEVELOPMENT OF LEYDIG CELL TUMOURS

1.7.1. Occurence

Tumours of the testis represent only 0.5 to 2% of all tumours in men (Davis *et al.*, 1980). These tumours may originate from either germ cells or somatic cells, although as shown in Table 1.1, the vast majority of these tumours arise from germ cells (Skakkebaek *et al.*, 1984; Matsumoto, 1988).

With the exception of the spermatocytic seminoma, all germ cell tumours originate from the intratubular undifferentiated carcinoma-in-situ germ cell. The differentiation into the different tumour types may take place inside the seminiferous tubules or after the germ cell has invaded the interstitial tissue (Skakkebaek *et al.*, 1984).

In the human, Leydig cell tumours of the testis account for 1 to 3% of all testicular tumours (Dixon & Moore, 1953; Mahon *et al.*, 1973; Mostofi & Price, 1973; Audi *et al.*, 1984; Skakkebaek *et al.*, 1984), with 10% being malignant and almost 10% being bilateral (Dalgaard & Hesselberg, 1957; Mahon *et al.*, 1973). These tumours produce a wide variety of steroid hormone changes leading to a spectrum of symptoms, including precocious puberty (Kay *et al.*, 1975), gynecomastia, change in libido, and oligospermia (Mineur *et al.*, 1987). About 25% of Leydig cell tumours in adult men secrete predominantly oestrogens, which result in gynecomastia (Hunt & Budd, 1939; Gabrilove *et al.*, 1975; Sohval *et al.*, 1977; Castle & Richardson, 1986; Corrie *et al.*, 1987; Valensi *et al.*, 1987). The feminizing neoplasms are usually benign (Gabrilove *et al.*, 1975).

Histological type	Occurence (%)
Seminoma	39.5
Non-seminoma	36.6
Combined (seminoma + non-seminoma)	13.5
Malignant lymphoma	6.7
Sertoli cell tumour	1.2
Leydig cell tumour	1.9
Miscellaneous	3.6

Table 1.1. Distribution of testicular tumours according to histological type (Skakkebaek *et al.*, 1984)

Similar to the human, spontaneous Leydig cell tumours occur infrequently in most strains of laboratory rats (Thompson *et al.*, 1961; Snell, 1965). However 78% of Wistar male rats of the substrain-U (U-rats) (Teerds *et al.*, 1991) and over 95% of Fischer male rats exhibit some degree of interstitial cell neoplasia (Turek & Desjardins, 1979; Barke *et al.*, 1985; Morii *et al.*, 1988). Nodular interstitial cell hyperplasia, observed in some animals by 10 months of age, was found to precede interstitial cell neoplasia which became evident at about 12 and 17 months of age in U-rats and Fischer rats respectively (Coleman *et al.*, 1977; Turek & Desjardins, 1979). In mice, spontaneous testicular tumours are extremely rare and most of the reported instances are of Leydig cell origin. In inbred H mice, which have a high incidence of breast tumours in the male, lesion of the testes, including Leydig cell hyperplasia and tumours were found with an incidence of 1% (Mostofi & Bresler, 1979).

Leydig cell tumours are also found in the ovary in the form of Sertoli-Leydig cell tumours (SLCT). They account for less than 0.5% of ovarian tumours and represent the most frequent ovarian virilizing tumour which is often accompanied by defeminization (e.g. oligo- and amenorrhea) (Novak & Long, 1965; Scully, 1977; Haider *et al.*, 1985; Talerman, 1987; Mango *et al.*, 1988). The ovarian SLCT are sex cord stromal tumours that exhibit a testicular direction of differentiation. These tumours exhibit a wide range of patterns. The main histological observations of the SLCT tissue include tubular structures, solid nests of "Sertoli cells" and islands of "Leydig cells" (Genton, 1980; Haider *et al*, 1985). The strongest evidence for the origin of ovarian

Sertoli and Leydig cells given by Genton (1980) supports the view that the SLCT arises from ovarian stroma. The SLCT produce mainly testosterone and to a lesser degree, androstendione, progesterone, oestrone, and 17α-hydroxyprogesterone (Haider *et al.*, 1985; Mango *et al.*, 1988).

1.7.2. Effects of cryptorchidism

The risk of Leydig cell tumours was found to be raised in men with a history of cryptorchidism (Chilvers *et al.*, 1987; Swerdlow *et al.*, 1987; Palmer, 1991). The incidence increases with the degree of cryptorchidism and is highest in patients with abdominal testes (Palmer, 1991). Damage of the intratubular compartment of seminiferous tubules as a result of cryptorchism was found to be associated with Leydig cell hyperplasia and loss of luteinizing hormone receptors in the affected testis in Sprague Dawley rats (Risbridger *et al.*, 1981). In contrast, in Fischer rats, surgical cryptorchidism prevented the development of Leydig cell tumors in the aged rats as did the chronic administration of diethylstilbestrol (Huseby, 1981)

1.7.3. Morphological changes which precede the development of Leydig cell tumours

Leydig cell tumours originate as hyperplasia of the Leydig cells, which then can progress to a neoplasia (Mostofi & Bresler, 1979; Houseby, 1981; Amador *et al.*, 1985). Histologically, the commonly seen picture of Leydig cell tumours is that of polygonal cells with vacuolated cytoplasm and a round or oval nucleus. Binucleated and multinucleated cells are common (Sohval *et al.*, 1977a; Mostofi & Bresler, 1979; Grem *et al.*, 1986; Morii *et al.*, 1988). The nuclear

membranes tend to be irregular and undulating and no distinguishing ultrastructural characteristics were discerned between feminizing and virilizing Leydig cell tumours (Sohval *et al.*, 1977a). Hyperplastic cells have abundant eosinophilic cytoplasm and discrete cell borders. Neoplastic cells are pleomorphic and often spindloid and have fibrillar, finely vacuolated cytoplasm (Gelberg & McEnter, 1987).

Two types of Leydig cells were often found within the tumour tissue; large cells which are histologically identical to the nodular Leydig cells, and small cells with a more elongated nucleus and scanty cytoplasm (Mostofi & Bresler, 1976; Turek & Desjardins, 1979; Morii *et al.*, 1988; Teerds *et al.*, 1991).

The diagnosis of malignancy is difficult to make; increased mitosis, vascular invasion and local extension of the tumour should suggest malignancy, but the best criterion is metastasis or transplantability of the tumour (Mostofi & Bresler, 1979; Gelberg & McEnter, 1987).

The histogenesis of Leydig cell tumours after administration of oestrogens has been described in mice. The interstitial cells first hypertrophy and degenerate. New Leydig cells then differentiate from interstitial mesenchyme in various foci giving rise to nodules, which eventually form tumours which resemble those which occur spontaneously in the susceptible strains of mice and rats (Mostofi & Bresler, 1979; Houseby, 1981).

A morphological study by Teerds *et al.* (1991) using U-rats suggests that the Leydig cell tumours develop from a rapid, focal outgrowth of a Leydig cell nodule and not as a result of a gradual and diffuse increase in the number of

Leydig cells. This was supported by the fact that at the periphery of the tumour, compression of the seminiferous tubules was found, while within the tumour tissue no remnants of entrapped seminiferous tubules could be detected. The latter pattern being characteristic for diffuse hyperplasia (Mostofi & Bresler, 1976).

In general, larger tumours were accompanied by severe atrophy of the surrounding seminiferous tubules, while smaller ones were found within the active testicular tissues (Morii *et al.*, 1988). Involution of the seminiferous epithelium in all testes exhibiting extensive interstitial cell proliferation has been found in Fischer rats (Brown *et al.*, 1979; Turek & Desjardins 1979; Bartke *et al.*, 1985) and in the human (Mineur *et al.*, 1987). The tubular atrophy was found to be confined to regions of the testis containing nodules of hyperplastic Leydig cells (Brown *et al.*, 1979). This was attributed to the regional reduction in the availability of testosterone to the germinal epithelium, progesterone-mediated suppression of gonadotrophin release, and/or to the disruption of suspected local paracrine relationship between the germ cells, the Sertoli cells and the Leydig cells (Bartke *et al.*, 1985). The intertubular spaces often appeared oedematous and contained thick-walled arteriols (Brown *et al.*, 1979).

1.7.4. Changes in hormone levels which precede the development of Leydig cell tumours

In several instances, the development and maintenance of testicular tumours has been shown to coincide with the changes in gonadotrophins and/or gonadal

hormones associated with ageing thereby increasing the incidence of Leydig cell tumours (Turek & Desjardins, 1979; Bartke *et al.*, 1985; Morii *et al.*, 1988). Plasma testosterone levels decline with age in male rats (Chan *et al.*, 1977; Gray, 1978; Harman *et al.*, 1978). However, unlike ageing men (Rubens *et al.*, 1974; Hallberg *et al.*, 1976), the aged male rat does not undergo a gradual elevation of plasma LH levels (Riegle & Meites, 1976; Gray, 1978). Instead, declining plasma LH levels with advancing age have been noted in strains of rats including Wistar, Long-Evans (Gray, 1978; Shaar *et al.*, 1975; Reigle & Meites, 1976), Wistar-U (Teerds *et al.*, 1991), and Fischer (Amador *et al.*, 1985) rats. However, a study by Turek & Desjardins (1979) using Fischer rats did not show any decrease in serum testosterone or LH but a transient increase in serum LH at 18 months of age was observed. Also Sprague Dawley rats showed an increase in circulating LH levels and no significant change in circulating testosterone levels or in basal and gonadotrophin-stimulated production of this steroid by decapsulated testes (Kaler & Neaves, 1981).

In the human, there is no clear picture of the changes in circulating oestradiol, testosterone, LH and FSH in patients with Leydig cell tumours. Davis *et al.* (1980) reported a decrease in basal testosterone levels and a minimal increase in LH, FSH, and oestradiol. Grem *et al.* (1986) showed no change in testosterone and LH levels while FSH was elevated and Valensi *et al.* (1987) reported an elevated but variable levels of plasma oestradiol and decreased plasma LH, FSH, and testosterone.

Enzyme deficiencies in testosterone biosynthesis have been described in Leydig cell tumours from a patient with feminizing Leydig cell tumours (Bercovici *et al.*, 1981). The 17-ketosteroid-reductase (Audi *et al.*, 1984) and 3ß-HSD (Lipsett *et al.*, 1966) deficiency were reported to occur in patients with a Leydig cell tumour(s). In aged Fischer rats with spontaneous Leydig cell tumours, there is an alteration in the testicular testosterone synthesizing pathway at a step after progesterone (Amador *et al.*, 1985; Bartke *et al.*, 1985). In addition, oestrogen which is implicated in the development of Leydig cell tumours, was found to cause a defect in 17α-hydroxylase and C17-20 lyase activity (Jones *et al.*, 1978; Forest *et al.*, 1979; Bercovici *et al.*, 1981; Mineur *et al.*, 1987).

1.7.5. Induction of Leydig cell tumours

The endocrine stimuli most centrally involved in the genesis of Leydig cell tumours are quite different in the mouse and the rat; an abnormal response to gonadotrophin stimulation being pivotal in the rat while reaction to oestrogens is the most significant factor in the mouse (Huseby, 1981).

1.7.5.1. Induction of Leydig cell tumours in the mouse

The commonest effective method of inducing Leydig cell tumours in mice is prolonged administration of oestrogenic compounds; however, a number of factors e.g. mouse strain, specific agent, and duration of treatment affect the results. Burrows (1935) first observed Leydig cell hyperplasia in oestrogen-treated strain A mice. BALB/c mice are more susceptible to Leydig cell tumour induction with oestrogen or other agents than other strains (R3, CBA,

C121, C3H strains) (Mostofi & Bresler, 1979). The effect of oestrogen on Leydig cell proliferation was strengthened by the observation that the rate of DNA synthesis was highest in Leydig cells isolated from oestrogen-treated BALB/c mice which have a high incidence of Leydig cell tumours compared with the controls and C3H/Bi mice, which have a low incidence of Leydig cell tumours (Spruance *et al.*, 1978).

Although oestradiol must be administered continuously for 3 to 5 months before Leydig cell hyperplasia occurs and for 7 to 9 months before malignant changes appear, morphological and biochemical changes occur much earlier (Kurland *et al.*, 1975). After 1 week of continuous oestrogen administration to mice with cryptorchid testes, there are marked changes in the enzymes of steroid biosynthesis (Samuels *et al.*, 1969). This effect was found to be exerted directly on the testis not through the pituitary, since a similar effect is observed in hypophysectomized mice treated with oestrogens (Huseby & Samuels, 1977). In addition, oestrogen was found to stimulate the growth of T124 958-R mouse Leydig tumour cell line *in vivo* and *in vitro* (Noguchi *et al.*, 1986). Also, the presence of oestrogen receptors in malignant mouse Leydig cells (Sato *et al.*, 1978; Zimniski *et al.*, 1985) and their involvement in the effect of oestrogen on cell proliferation was demonstrated by Sato *et al.* (1985).

In addition to oestrogens, Leydig cell tumours can be induced in mice by triphenylethylene, cryptorchidism, cadmium, cyclophosphamide, methylcholanthrene, and ethyl glycol (Mostofi & Bresler, 1979).

1.7.5.2. Induction of Leydig cell tumours in the rat

A possibility of a causal relationship between LH levels and development of Leydig cell tumours is strongly supported by results obtained by Brown *et al.* (1979) in parabiosed Fischer rats. In that study, chronic elevation of gonadotrophin levels produced by 20 months of parabiotic union with castrated males or ovariectomized females resulted in development of interstitial cell tumours in 11 of 20 animals. Furthermore, treatment with hCG (400 IU/rat) was found to result in Leydig cell hyperplasia three days after treatment in rats (Léon *et al.*, 1987). A similar effect was found in men who received 5,000 IU of hCG three times weekly for periods of forty seven to sixty-five days. In the latter study, an increase in urinary oestradiol excretion was found to be associated with an increase in Leydig cell number (Maddock & Nelson, 1952). However, Huseby (1981) demonstrated that parabiosis of Fisher rats with a castrate male partner which results in an increase in circulating levels of LH does not alter the process of Leydig cell tumour development.

Bartke *et al.* (1985) provided evidence that chronic elevation of peripheral prolactin levels in male Fischer rats, by transplantation of anterior pituitaries from adult females Fischer rats under the kidney capsules in early adult life or by treatment with oestrogens, was extremely effective in preventing the development of Leydig cell tumours. This was strengthened by a study using the same strain of rats in which a decrease in circulating prolactin levels associated with the presence of Leydig cell tumours was observed at 27 months of age (Amador *et al.*, 1985). However, a study by Turek & Desjardins

(1979) using the same strain of rats showed an increase in serum prolactin (PRL) levels with age.

1.7.5.2.1. The role of oestrogens in the induction of Leydig cell tumours by LH/hCG

The hyperplastic effect exerted by hCG in rats was attributed to oestradiol, since this treatment results in an increase in the circulating levels of this steroid and intratesticular administration of oestradiol benzoate was able to reproduce the tubular damage caused by hCG treatment which leads to Leydig cell hyperplasia (Léon *et al.*, 1987). The involvement of oestradiol in the development of Leydig cell tumours in the rat was supported by studies showing the association of Leydig cell tumours with high circulating levels of oestradiol (Turek & Desjardins, 1979; Mordes *et al.*, 1984; Orczyk *et al.*, 1987). However, treatment with oestrogens prevents the development of Leydig cell tumours in Fischer rats (Bartke *et al.*, 1985). This is probably due to the fact that oestrogen treatment results in an increase in plasma prolactin levels due to induction of lactotroph hyperplasia and thus prevent the development of Leydig cell tumours (Bartke *et al.*, 1985). Furthermore, hyperprolactinaemia results in a decrease in plasma LH (Bartke *et al.*, 1985) and thus prevent the increase in LH levels which lead to Leydig cell hyperplasia.

The local levels of oestradiol produced within the testis are likely to be more important in the induction of Leydig cell tumours than the circulating levels. Since only 24% of circulating oestradiol is of testicular origin (Payne *et al.*, 1976), the determination of plasma levels of this steroid does not reflect the

local concentration within the testis which determines the incidence of the development of Leydig cell tumours. Selective sampling of the spermatic vein of a testis containing a Leydig cell tumour(s) revealed high oestrogen levels compared with the circulating levels (Gabrilove *et al.*, 1975; Castle & Richardson, 1986; Corrie *et al.*, 1987). It was suggested that the pathophysiology of the development of Leydig cell tumours is based on a primary Leydig cell abnormality of oestrogen production which leads to the sequential development of Leydig cell hyperplasia and eventually tumour formation (Castle & Richardson, 1986). The disturbed intratesticular oestrogen/androgen concentrations exert their action through two different pathways, one occurs systematically, via the blood stream, affecting both testes and the other is a local mechanism involving direct influence on Leydig cells, Sertoli cells and germ cells (Sohval *et al.*, 1977b).

Studies related to the implication of oestradiol in the aetiology of human breast (O'Neil *et al.*, 1980; McNeil *et al.*, 1986; O'Neill & Miller, 1987; Miller & O'Neill, 1987; Lϕnning *et al.*, 1990) and prostatic (Seppelt, 1978; Ghanadian & Puah, 1981; Iqbal *et al.*, 1983; Stone *et al.*, 1986) cancer are in favour of the importance of the local concentrations rather than the circulating levels of oestradiol in the development of the tumours. Furthermore, E2-17ß HSD, which regulates tissue concentrations of oestradiol and thus exposure of the oestrogen receptor to this biologically active oestrogen, has been detected in breast cancer cells. In MCF-7 (oestrogen receptor positive) breast cancer cells, E2-17ß HSD reductive activity predominates, Whereas in MDA-MB-231

(oestrogen receptor negative) cells its oxidative function is more important (Pasqualini *et al.*, 1989).

1.7.6. The mechanism of tumour development

1.7.6.1. Involvement of paracrine factors

The mechanism of tumour development is unclear. Evidence of Leydig cell hyperplasia near the tumour (Bercovici *et al.*, 1981; Valensi *et al.*, 1987) suggests the existence of a factor stimulating Leydig cell proliferation. Seminiferous tubule and Sertoli cell involution has often been observed in the peritumoral testis (Brown *et al.*, 1979; Turek & Desjardins, 1979; Bercovici *et al.*, 1981; Bartke *et al.*, 1985; Mineur *et al.*, 1987). The resulting imbalance between Sertoli and Leydig cells could induce changes in Leydig cell function and multiplication (Risbridger *et al.*, 1981; Sharpe, 1983). Considering the interactions between the different types of cells within the testis (see Sharpe, 1990; Skinner, 1991 for review), it is not known if the mechanism by which this occurs is due to a lack of a controlling influence on interstitial cells by a nonfunctioning seminiferous tubule or whether damaged tubules produce an interstitial cell growth stimulator. There is evidence that Sertoli cells secrete factor(s) that stimulate germ cell (Kancheva *et al.*, 1990) and Leydig cell (Ojeifo *et al.*, 1990) proliferation. As intact seminiferous tubules secrete a factor which inhibits aromatase activity (Sharpe, 1983), tubular lesions could also contribute to the increase in intratumoral aromatase activity and thus local oestradiol levels which contribute to the development of Leydig cell tumours.

The mechanism by which interstitial cell proliferation occurs is most likely multifactorial and vary among species. This is not unexpected, since there are well-known differences among species in interstitial cell structure and function (Ewing *et al.*, 1979) and induction of Leydig cell tumours (Huseby, 1981).

Oestrogens have been implicated in enhancing both growth factor formation and hormonal receptor levels in endocrine target tissue (Beck & Garner, 1989), and such growth factor production via a paracrine mechanism may be involved in Leydig cell tumour aetiology. IGFs, particularly IGFII, are synthesized by many tumours. Many of the same tumours contain IGF-I receptors (Serio & Fiorelli, 1991).

Oestradiol induces expression of growth-related c-fos and c-myc proto-oncogenes (van der Burg *et al.*, 1989; Beck & Garner, 1989). The c-myc oncoprotein was found to be associated with the development of testicular tumours, since high levels of this protein were detected in seminoma and differentiated teratoma of the testis (Sikora *et al.*, 1985; Watson *et al.*, 1986). Protein tyrosine kinases, which are now recognized as having potentially important roles in the regulation of cell growth (Ek *et al.*, 1982; Earp *et al.*, 1983; Kasuga *et al.*, 1983; Jakobovits *et al.*, 1984), were found to be present at high levels in mouse Leydig tumour cells (Dangott *et al.*, 1986) and normal Leydig cells of the testis (Dangott *et al.*, 1983).

Prostaglandins are also implicated in tumour development; under certain conditions, PGE_2 can promote the growth of tumours by suppressing the immune system, and the use of prostaglandin synthesis inhibitors (aspirin,

indomethacin and flurbiprofen) has been shown to inhibit tumour growth in mouse fibrosarcoma (for review, see Smythies, 1988).

1.7.6.2. The role of peroxisomes in tumour development

The involvement of peroxisomes in the development of tumours is supported by studies on hepatocarcinogenesis caused by peroxisome proliferators (Reedy *et al.*, 1979; Styles *et al.*, 1988). In addition, large numbers of peroxisomes with diameter ranging from 0.1 to 0.4μm were found in rat Leydig cell tumours (Reddy & Svoboda, 1972b). A mouse peroxisome proliferator activated receptor (mPPAR), a novel member of the nuclear receptor superfamilly, which may be implicated in the process of tumour development has been recently cloned and characterized by Isseman & Green (1990). The mechanism by which peroxisome proliferators regulate gene expression and induce tumour development is unknown. It was suggested that these agents could induce the putative endogenous ligand of PPAR or may directly activate the receptor for example by phosphorylation (Issemann & Green, 1990). Of the mouse tissues examined, expression of mPPAR is restricted to those that are responsive to peroxisome proliferators (kidney > liver > heart). The expression in the testis and brain was found to be very low (Isseman & Green, 1990) and thus provides a possible explanation for the very low incidence of testicular tumours in mice. However, no data are available with respect to the expression of PPAR in the rat, human and other species in order to compare the difference in Leydig cell tumour induction in these species.

1.7.6.3. The possible involvement of inhibin in tumour development

Inhibin was also found to be implicated in the aetiology of tumour development; serum inhibin levels were abnormally elevated in patients with primary and recurrent granulosa cell tumours (Loppöhn *et al.*, 1989). This is supported by a study by Piquette *et al.* (1990) who demonstrated that inhibin-α and -ß subunits are produced and their mRNAs are expreseed in Equine granulosa-theca cell tumours. In addition, high serum levels of inhibin were found in a post-menaupausal women with a virilizing Sertoli-Leydig cell tumour, and this was found to be associated with high immunoreactive inhibin content in the tumour and low circulating levels of LH and FSH (Ohashi *et al.*, 1990). In male dogs in which testicular tumors are more common (5-12% of all neoplasms), Sertoli cell tumours contained increased concentrations of mRNA for the inhibin-α and specially, ßB subunit when compared with control testes. This was associated with increased bioactive inhibin content of the tumour and increased peripheral levels of immunoreactive inhibin (Grootenhuis *et al.*, 1990). Human Leydig cell tumours also produce immunoreactive inhibin (de Jong *et al.*, 1990). However, a study by Teerds *et al.* (1991) using Wistar U-rats did not show any change in circulating inhibin levels during the development of Leydig cell tumours.

1.7.6.4. The role of Macrophages in tumour development

The presence of infiltrating macrophages has often been observed in histological sections of certain types of human tumours and has been claimed to indicate good prognosis. Evans (1973) found that macrophage contents of

different experimental tumours were found to range from 4-56% of the total cell population. These cells were shown to be of host origin, most if not all being derived from circulating blood monocytes rather than by self-replication of macrophages within the tumour. Large numbers of macrophages were found in benign and malignant breast tissue with significantly higher numbers occuring in the malignant group tumours (Kelly *et al.*, 1988). A role for tumour macrophages in the control of tumour dissemination was suggested, as spontaneous metastasis was found to be associated with a decrease in macrophage infiltration (Eccles & Alexander, 1974). Furthermore, Fidler (1976) demonstrated that i.v. injection of macrophages treated *in vitro* with supernatants from cultures (containing lymphocytes from syngeneic mice, sensitized allogenic mice or sensitize rats) into mice significantly reduced the number of established metastasis. The synthesis of TNF, a factor secreted by macrophages and involved in their cytotoxic effect, is decreased in aged (Bradley *et al.*, 1989; Davila *et al.*, 1990) and hypophysectomized rats (Edwards III *et al.*, 1991). Growth hormone was found to increase the synthesis of TNFα by macrophages, an effect mediated by an increase in the synthesis and secretion of interferon-gamma (INF-gamma) by T-lymphocytes or natural killer cells (Edwards III *et al.*, 1991). Also, Interleukin-2 (IL-2) stimulates the production of TNF by macrophages (Economou *et al.*, 1989).

1.8. EFFECTS OF DOPAMINE AND ITS AGONISTS ON THE HYPOTHALAMIC-PITUITARY-ADRENAL AND GONADAL AXIS

Dopamine plays a major role in numerous physiological functions in the periphery as well as in the central nervous system. In particular, dopamine-responsive cells are clearly implicated in the etiology and/or treatment of disorders such as schizophrenia and Parkinson's disease (Seeman & Niznik, 1990). Each of these diseases may be treated with agents that interact with dopamine receptors (Seeman, 1976; Baldessarini, 1985).

1.8.1. Dopamine receptors

Two distinct types of dopamine receptors (D1 and D2) have been identified on the basis of pharmacological, biochemical, and functional criteria (Kebabian & Calne, 1979). D1 receptors activate adenylate cyclase (AC) and are coupled with the Gs regulatory protein. By contrast, activation of D2 receptors results in various responses including inhibition of AC, inhibition of phosphatidylinositol turnover, increase in K^+ channel activity and inhibition of Ca^{2+} mobilization (Vallar, 1988; Andersen *et al.*, 1990) (Figure 1.10). Furthermore, recent studies are suggestive of the presence of a non-adenylate cyclase-coupled D1 receptor subtype (Andersen *et al.*, 1990). However, this concept was rejected after cloning of the D1 receptor (for review, see Sibley, 1991). Dopamine receptor types D1 and D2 can oppose or enhance each other's actions for electrical, biochemical, and psychomotor effects (Walters *et al.*, 1987; Seeman *et al.*, 1989). In the anterior pituitary, there is no D1 receptor, while in the striatum there are links between D1 and D2 receptors (Seeman *et al.*, 1989).

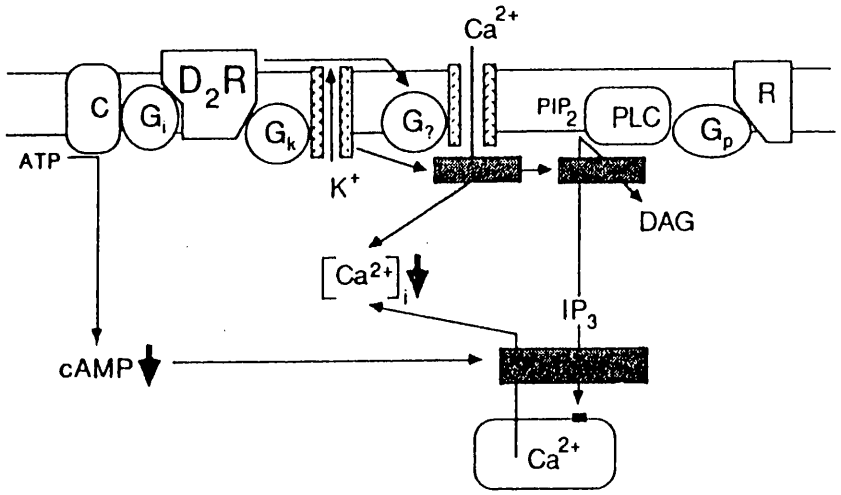


Figure 1.10. Model of signal transduction at the D2 receptor.
The receptor (D2R) is coupled via G proteins (G_i, G_k, and $G_?$) to adenylate cyclase (AC). K^+ channels and possibly Ca^{2+} channels. D2 receptor activation interferes (shaded bars) with the PIP_2-IP_3 pathway by two mechanisms: the decrease in cAMP levels blocks the IP_3-induced Ca^{2+} release from intracellular stores; the lowering of sustained $[Ca^{2+}]_i$ increase via Ca^{2+} channels causes a late inhibition of PIP_2 hydrolysis triggered by stimulatory receptors (R) (Vallar & Meldolesi, 1989).

The dopamine D2 receptor is found presynaptically as well as postsynaptically. Functionally, the presynaptic D2 receptor (i.e. autoreceptor) inhibits synthesis and release of dopamine. This receptor appears not to be coupled to adenylate cyclase, but little is known about its signal transduction pathway (Andersen *et al.*, 1990).

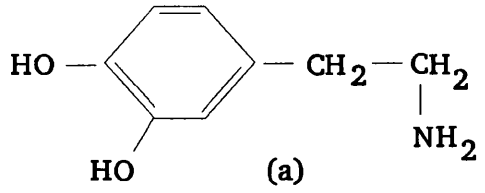
Activation of D2 receptors mediates inhibition of the activity of various cell types, including striatal neurones (Kebabian & Calne, 1979), pituitary lactotrophs (Stoof & Kebabian, 1984; Niznik, 1987) and melanotrophs (Kebabian *et al.*, 1972; Brown *et al.*, 1977; DeCamilli *et al.*, 1979; Munemura *et al.*, 1980). The diversity of responses elicited by D2-receptor activation reflects the existance of multiple D2 receptor subtypes, the identification of which is facilitated by the recent cloning of a cDNA encoding a rat D2 receptor which indicates that it belongs to the large superfamily of receptors coupled to G proteins and its topology includes seven transmembrane domains (Dohlman *et al.*, 1987; Bunzow *et al.*, 1988). In this family, the genes are frequently without introns and each is believed to encode a unique polypeptide product. However, the gene for the D2 receptor produces two receptor isoforms by alternative mRNA splicing; one isoform corresponds to the D2(415aa) receptor, but the second contains an additional sequence encoding a 29 amino-acid fragment, defining a novel D2(444aa) receptor isoform (Grandy *et al.*, 1989; Giros *et al.*, 1989; Monsma *et al.*, 1989; Chio *et al.*, 1990; for review, see Sibley, 1991). Expression of the two isoforms is tissue-specific, and both are regulated by guanyl nucleotides. As the extra sequence is located within a

putative cytoplasmic loop that binds to G proteins, it was suggested that the two isoforms might interact with different G proteins and thereby initiate distinct intracellular signals (Giros *et al.*, 1989). In addition to D1 and D2 dopamine receptors, a D3 dopamine receptor which is highly related to the D2 subtype has been recently characterized (for review, see Sibley, 1991). It differs in its pharmacology and signalling system from the D1 or D2 receptor and represents both an autoreceptor and a postsynaptic receptor. The D3 receptor is localized in limbic areas of the brain (tubercule > hypothalamus > substantia nigra), which are associated with cognitive, emotional and endocrine functions. It seems to mediate some of the effects of antipsychotic drugs and drugs used against Parkinson's disease, that were previously thought to interact only with D2 receptors (Sokoloff *et al.*, 1990).

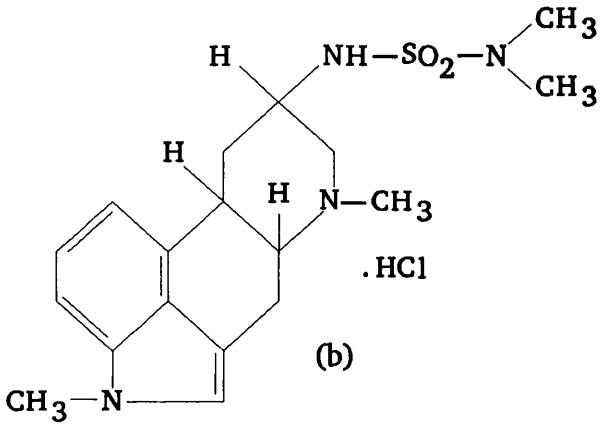
The concept of multiple categories of dopaminergic receptors has important implications for therapeutic medicine. Drugs which modify activity at the D2 receptor have been exploited to treat endocrinological, neurological and psychiatric disorders.

1.8.2. Pharmacology and clinical application of mesulergine

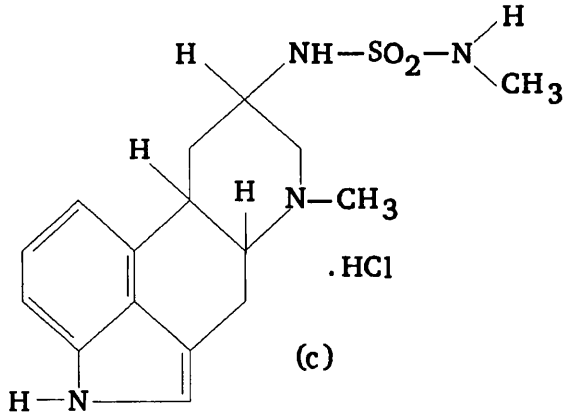
Mesulergine (1,6-dimethyl-8α-N,N-dimethyl sulfamoyl amino-ergoline, CU 32-085) (Figure 1.11) was developed for use in man in the treatment of Parkinson's disease and hyperprolactinaemia (Jelinger, 1982). In rats, after oral (p.o.) or intraperitoneal (i.p.) administration, mesulergine exerts effects typical of dopamimetic drugs: (1) inhibition of prolactin secretion, (2) reduction of striatal dopamine turnover, and (3) suppression of exploratory behavior in mice.



(a)



(b)



(c)

Figure 1.11. Chemical structure of dopamine (a), mesulergine (CU 32-085) (b), and the 1,20-N,N-bidemethylated metabolite of mesulergine (Markstein, 1983).

However, after subcutaneous (s.c) application, mesulergine exhibits a biphasic, opposing effects (Enz, 1981; Enz *et al.*, 1984; Markstein, 1983). Initially, it increases dopamine turnover and antagonizes the effect of apomorphine on ɣ-butyrolactone-induced elevation of dopamine synthesis, indicating dopamine antagonistic effects. After few hours, a decrease in dopamine turnover and a reversal of gamma-butyrolactone-induced stimulation of tyrosine hydroxylase are observed, which is consistent with agonistic effects at dopamine receptors. Since N-demethylated metabolites of mesulergine have been found after its administration to rats, dogs, and monkeys, the dopamimetic effects of this compound were attributed to the pharmacologically active metabolites formed *in vivo* (Enz, 1981; Enz *et al.*, 1984; Marko, 1984). This was confirmed by *in vitro* studies using pituitary cell culture preparations; the N-1 demethylated metabolite, CH 29-717, inhibited prolactin release while mesulergine was only weakly active even at high concentrations and counteracted the effect of the metabolite when incubated together (Marko, 1984). The substitution at N-1 of the lysergic moiety of mesulergine is responsible for the dopamine-antagonistic effect (Enz *et al.*, 1984). When administered to animals, the metabolites of mesulergine were found to concentrate in the striatum than in the plasma (Enz *et al.*, 1984) suggesting the mostly central effect of mesulergine.

Both mesulergine and the bidemethylated metabolite interact with D1- and D2-receptors. However, in view of the high concentrations required to affect D1 receptors, only the effect at D2 receptors appear to be relevant to explain the *in vivo* effects of these drugs.

1.8.3. Effects of dopamine and its agonists on anterior pituitary hormones

The dopamine agonistic effects of mesulergine when given to rats orally, include inhibition of PRL release, an increase in circulating levels of LH and ACTH, and a decrease in growth hormone (GH) (when given for > 4 weeks) (Siegel & Prentice, 1987). Similar effects were found when rats were treated with dopamine or other agonists (e.g. bromocriptine, lisuride, lergotrile) (Baird *et al.*, 1985; Denef, 1988; Kebabian & Calne, 1979; Kerulich, 1979; Simpkins *et al.*, 1977; Vallar *et al.*, 1988)

1.8.3.1. Effects of dopamine and its agonists on prolactin synthesis and release

It is generally accepted that PRL release is regulated primarily by tonic inhibitory influence of dopamine (Beck & Wuttke, 1977; Judd *et al.*, 1978) acting at lactotroph D2 receptors (Godsmith *et al*, 1979; Kebabian & Calne, 1979; Baird *et al.*, 1985). D_2 receptors are negatively linked to various second messenger pathways, including adenylate cyclase, phosphoinositide turnover and intracellular calcium mobilization (Figure 1.9) (Vallar *et al.*, 1988; Lambert & McLeod, 1990), via several G proteins (Senogles *et al.*, 1987; Bouvier *et al.*, 1991a). In addition, dopamine inhibits the transcription of the PRL gene in rat pituitary cells; PRL 5'-flanking DNA was found to contain a dopamine response element(s) which can respond to dopaminergic signals mediated by either D_2 receptor isoform (Mechesney *et al.*, 1991). In addition to the hypothalamus, there is evidence supporting the participation of the posterior pituitary gland in determining the final concentration of dopamine acting at the lactotroph (Ben-Jonathan, 1985). Most of the axons of the dopaminergic neurons of the

arcuate nucleus end in the median eminence, but some are directed to the posterior lobe (Bjorklun *et al.*, 1973). Dopamine can reach the anterior pituitary lactotrophs from the posterior lobe via the short portal vessels (Page & Bergland, 1977). Furthermore, there is evidence that the posterior pituitary contains a potent PRL-releasing factor (Hyde & Ben-Jonathan, 1989) which might exert its effect by inhibiting DA release and/or synthesis.

In normal and tumorous rat lactotrophs, bromocriptine and other dopamine agonits primarily inhibit PRL secretion (MacLeod & Lehmeyer, 1974; MacLeod, 1976). Prolactin synthesis and mRNA concentrations are secondarily suppressed (Maurer, 1980a, Vidal *et al.*, 1988), while an increased intracellular degradation of newly synthesized PRL within lactotrophs is observed (Dannies & Rudnick, 1980; Maurer, 1980b).

Very long-term bromocriptine therapy has become the primary modality of therapy of prolactinoma patients. To optimize life long therapy of prolactinoma patients with dopamine agonists, new compounds that have a longer period of action and that may be better tolerated are currently under investigation (Franks *et al.*, 1981; Kleinberg *et al.*, 1980; Ferrari *et al.*, 1986; Melis *et al.*, 1987; Rasmussen *et al.*, 1987; Vidal *et al.*, 1988; Ciccarelli *et al.*, 1989; Gaillard *et al.*, 1989; Lee vance et al., 1989; Khalfallah *et al.*, 1990, Serri *et al.*, 1990). Besides its prolactin inhibitory action, the dopamine agonist (CV 205-502) transiently stimulates growth hormone secretion (Miell *et al.*, 1990), but has no effect on the other anterior pituitary hormones (Gaillard *et al.*, 1989).

The other inhibitory factors involved in the regulation of prolactin release and/or synthesis include GABA, somatostatin, gonadotrophin-releasing hormone-associated peptide (GAP), and autoregulation by prolactin at the level of the lactotroph (for Refs, see Lamberts & McLeod, 1990).

In addition to the feedback effect of peripheral PRL on the activity of the hypothalamic dopaminergic neurons (HDN), a variety of neurotransmitters and neuropeptides have been implicated in affecting the amount of dopamine released into the portal circulation and/or stimulating simultaneously the release of hypothalamic PRL release-stimulating factors. Opiates are a class of neurotransmitters that exerts a powerful influence on prolactin secretion. Both morphine and ß-endorphin stimulate PRL release by inhibiting the synthesis and release of dopamine by the HDN whereas naloxone atenuates these effects.

Although PRL-releasing activity can be demonstrated for a large number of neuroendocrine substances, the best understood and most commonly studied are the neuropeptides TRH, angiotensin-II (AII), neurotensin (NTS), bombesin (BBS), and vasoactive interstinal peptide (VIP). TRH, AII, NTS, BBS all act through an enhancement of polyphosphoinositide hydrolysis. In contrast VIP acts through increased cAMP (for Refs, see Jarvis *et al.*, 1988; Lamberts & MacLeod, 1990).

The direct effects of inhibitory and stimulatory hypothalamic factors on the lactotroph are modified by peripheral hormones that reach the anterior pituitary gland via systemic circulation. These include oestrogens which exert

a stimulatory effect on the synthesis and release of prolactin (Saade *et al.*, 1989; Tong *et al.*, 1989). In the rat there are at least three mechanisms by which oestrogens stimulate prolactin secretion; 1) a direct effect at the pituitary level by stimulating PRL synthesis, storage, and secretion, 2) modulation of hypothalamic PRL-inhibitory and stimulatory factors, and 3) an alteration in the pituitary responsiveness to PRL-regulating factors (for review, see Lamberts & MacLeod, 1990). The later mechanism is supported by the observations reported by Raymond *et al.* (1978), West & Dannis (1980), and Munemura *et al.* (1989) in which oestrogen reversed the ability of dopaminergic agonists to inhibit prolactin release by uncoupling the D2 receptor-G-protein interactions in the lactotroph. In addition, Bouvier *et al.* (1991b) have recently demonstrated that oestrogens have a significant and specific inhibitory effect on pituitary G protein levels which may modulate the secretion of pituitary hormones such as PRL. In contrast to the rat pituitary cells, oestrogen-exposed primate pituitary cells are significantly more sensitive to the inhibitory effect of dopamine on PRL release. The other circulating hormones which are involved in the regulation of prolactin include thyroid hormone (T_3) and glucocorticoids which inhibit the synthesis of PRL (for Refs, see Lamberts & MacLeod, 1990).

1.8.3.2. Effects of dopamine and its agonists on LH secretion

In contrast to the central noradrenergic system which is the major activator of LH secretion (Vijayan & McCann, 1978) (Figure 1.12), the role of the dopaminergic system in the regulation of LH secretion is controversial. Intraventricular injections of dopamine were shown to stimulate LH secretion in the ovariectomized-oestrogen primed female rat (Vijayan & McCann, 1978) and dopamine also stimulated LHRH release from hypothalamic fragments *in vitro* (Rotszstein *et al.* 1976; Negro-Vilar, 1979). There is, however, considerable evidence that the central dopaminergic system inhibits LHRH and LH release. Both infusion of dopamine and administration of L-dopa or dopamine receptor agonists reduced LH levels in normal women (Le Blanc *et al.*, 1976; Beck & Wuttke, 1977; Lachelin *et al.*, 1977; Judd *et al.*, 1978; Ferrari *et al.*, 1981; Levinson *et al.*, 1985; Barnes *et al.*, 1986; Peter *et al.*, 1986; Barnes *et al.*, 1987) and in intact or ovariectomized rats (Mueller *et al.*, 1976; Droura & Gallo, 1977). Furthermore administration of a dopamine antagonist, metaclopramide, can stimulate LH secretion in both hypothalamic amenorrhoeic patients (Quigley *et al.*, 1980) and normal women during the midluteal phase of their cycles (Ropert *et al.*, 1984). However, chronic administration of antidopaminergic drugs have no effect (DeLeo *et al.*, 1989) or inhibits (Melis *et al.*, 1988) LH secretion in postmenopausal women. In addition, *in vitro* studies by Rasmussen *et al.* (1986) showed that dopamine can stimulate release of LHRH from the adult human hypothalamus by a dopamine receptor-mediated mechanism.

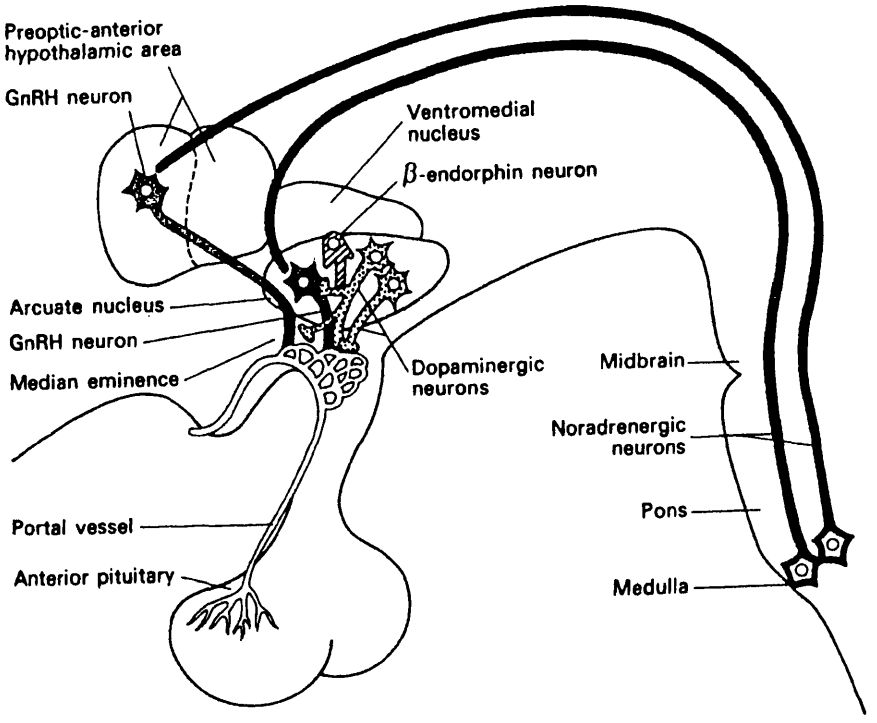


Figure 1.12. Schematic diagram showing some of the postulated neurochemical interactions which may control GnRH secretion (Johnson & Everitt, 1984).

In contrast to the direct effect on PRL release, dopamine and its agonist have been found to have an indirect effect on LH release; an effect through LHRH by decreasing the levels of this hormone was suggested (Schneider & Mc Cann, 1969; Beck & Wuttle, 1977; Kalra & Kalra, 1983; Barralough *et al.*, 1984; Peter *et al.*, 1986). These effects could be exerted through opioids, since a D2 receptor antagonist, veralipide, caused an increase in endogenous opioid levels in the hypothalamus and pituitary associated with a decrease in LH secretion (Melis *et al.* (1988). In addition opioids are also known to increase PRL and to decrease LH release (Pfeiffer *et al.*, 1987). However results from studies on humans, rabbits and ewes are strongly suggestive of a direct effect of dopamine and its agonist on LH release and synthesis (Peter *et al.*, 1986; Klibanski *et al.*, 1988; Kwekkeboom *et al.*, 1990). These effects have been found to involve D2 receptors, since they were blocked by using selective D2 receptor antagonists (e.g. sulpiride, metoclopramide and substituted benzamides such as veralipide) (Kebabian & Calne, 1979; Munemura *et al.*, 1980; Cote *et al.*, 1981; Baird *et al.*, 1985; Bunzow *et al.*, 1988).

The mechanism(s) by which the DA causes an increase in circulating levels of LH is not clear. It is possible that this is a result of:

1) a decrease in the number of LH receptors caused by the suppression of PRL release which leads to a decrease in Leydig cell function. This might results in an imbalance in the hypothalamo-pituitary-gonadal axis, whereby reduced Leydig cell responsiveness to LH would be compensated by chronically elevated LH secretion.

2) its inhibitory effect on PRL release/synthesis. Since, when present in high levels, PRL was found to inhibit basal and LHRH-stimulated LH release in the rat (Tresguerres *et al.*, 1981; Cheung, 1983; Sakar & Yen, 1985; Kooy *et al.*, 1989). Also, in ageing male rats, increased serum PRL levels were found to be associated with lowered gonadotrophins and a decrease in dopamine synthesis (Riegle & Meites, 1976; Simpkins *et al.*, 1977). 3) its direct effect on LHRH neurons. Since, in the rat, there is evidence that dopamine acts on α-adrenergic receptors to cause discharge of LHRH (Schneider & Mc Cann, 1969).

1.8.3.3. Effects of dopamine and its agonists on growth hormone secretion

The role of the central dopaminergic system in the regulation of GH has been studied extensively and the evidence is rather conclusive that in primates it is stimulatory to GH secretion. However, conflicting results were obtained in rats; Collu *et al.* (1973) and Kato *et al.* (1973) reported an inhibitory effect whereas Mueller *et al.* (1976) reported a stimulatory effect. However, Kitajima *et al.* (1989) reported a stimulatory effect of dopamine on both GHRH and somatostatin and the GHRH-stimulating effect of dopamine remains masked unless the action of somatostatin on GHRH neurons is eliminated.

1.8.4. Effect of ageing on the hypothalamic-pituitary-adrenocortical and gonadal axis: alteration in the responsiveness to dopamine and its agonists

Ageing-related changes occur in hypothalamic-pituitary-gonadal and adrenocortical control systems (Riegle & Meites, 1976; Flückiger *et al.*, 1983).

Ageing male rats show increased basal serum prolactin levels together with lowered gonadotrophins (Riegle & Meites, 1976; Simpkins *et al.*, 1977). The decrease in serum prolactin in response to L-dopa treatment was found to be less pronounced in aged compared with the young Long-Evans rats (Riegle & Meites, 1976). A decrease in dopamine and an increase in serotonin synthesis, which may be related to the changes in gonadotrophins and PRL, occur in the hypothalamus of old male Wistar rats (Simpkins *et al.*, 1977).

1.8.5. Effects of prolactin on Leydig cell function

Several reports have suggested that PRL plays a role in the modulation of Leydig cell function probably by potentiation of LH-stimulated responses (Hafiez *et al.*, 1972; Klemcke *et al.*, 1990). This modulatory role of PRL could be exerted through the control of the number of LH receptors (Zipf *et al.*, 1978; Morris & Saxena, 1980; Chan *et al.*, 1981; Klemcke & Bartker, 1981). The direct effect of PRL on the Leydig cell is supported by the demonstration of specific PRL testicular binding sites (Aragona & Friesen, 1975; Barkey *et al.*, 1977; Charreau *et al.*, 1977; Costlow & McGuire, 1977; Morris & Saxena, 1980; Katikinemi *et al.*, 1981; Chan *et al.*, 1981; Erichsen *et al.*, 1984; Bonifacio & Dufau, 1985). The PRL receptors represent a single class of high affinity sites (Ka= 8.7 nM) with a low capacity (980 sites per Leydig cell) (Barkey *et al.*, 1987). They are down regulated by PRL itself, testosterone and oestrogen (Barkey *et al.*, 1979; Morris & Saxena, 1980), LH (Davis *et al.*, 1980; Katikinemi *et al.*, 1981), and by GnRH (Catt *et al.*, 1979).

Testicular receptor sites for both LH and PRL undergo a common process of

increased availability during the early phase of Leydig cell activation by gonadotrophins. This transient increase in both binding sites was found to be followed by subsequent period of depletion that is short-lived for the PRL sites and prolonged for the LH receptors (Chan *et al.*, 1981). Rat tumour Leydig cells from a transplantable rat Leydig cell tumour (H-540) were found to have a reduced LH and PRL receptors compared with the normal Leydig cells with no change in their affinity (Erichsen *et al.*, 1984).

In addition to the positive effects of physiological levels of PRL, there have been many reports on the deleterious effects of hyperprolactinaemia at the level of the hypothalamic-pituitary-gonadal axis. In adult rats, hyper-prolactinaemia, induced by grafting pituitary under the kidney capsule or after inoculation of the PRL secreting tumour, was associated with reduced basal and LHRH-stimulated LH levels and reduced serum levels and the production of testosterone in response to hCG (Sharpe & McNeilly, 1979; Tresguerres *et al.*, 1981; Cheung, 1983; Sarkar & Yen, 1985; Kooy *et al.*, 1989).

1.8.6. Direct effect of dopamine and its agonists on the testis

Although there is no evidence for a direct effect of dopamine and its agonists on testicular function, dopamine receptors have been identified in the adrenal cortex of various mammalian species, including man (Dunn & Bosmann, 1981; Bevilacqua *et al.*, 1982; Missale *et al.*, 1985; Fraser *et al.*, 1989). Dopamine exerts a direct inhibitory effect on steroid secretion in frog adrenocortical cells (Morra *et al.*, 1989, 1990). This effect was found to involve dopamine D2 receptor-coupled to phospholipase C (PLC) and a pertussis toxin-sensitive G-

protein (Gi) associated with an inhibition of inositol phosphate and arachidonic acid formation (Morra *et al.*, 1991).

1.8.7. Effects of dopamine agonists on Leydig cell proliferation

During the safety assessment of a dopamine agonist (mesulergine, CU 32-085), it was found that the drug increased the incidence of Leydig cell tumours in a 2 ½ year carcinogenic study in rats but not in mice (Siegel & Prentice, 1987). This affect was attributed to the increase in serum concentration of LH. A number of pharmaceutical compounds such as cimetidine (Brimblecombe & Leslie, 1984), a calcium channel blocker (SDZ 200-110) (Roberts *et al.*, 1989), and the dopamine agonists (CF 25-397, an ergoline and AY 27-110, a nonergoline) (Siegel & Prentice, 1987) have also been shown to induce Leydig cell tumours in long-term studies in rats. Similar to mesulergine, SDZ 200-110 does not produce gonadal tumours in CD-1 mice of either sex or in female Sprague Dawley rats (Roberts *et al.*, 1989). Also the increase in the incidence of Leydig cell tumours in rats treated with this compound was attributed to the increase in serum levels of gonadotrophins which preceded the appearance of these tumours.

The mechanism by which SDZ 200-110 elevates serum gonadotrophin levels is not known but it was suggested that this may be related to the ability of the drug to inhibit the biosynthesis of testosterone while decreasing testicular LH receptors and by decreasing serum prolactin levels (Roberts *et al.*, 1989). Prolactin has been implicated in the control of gonadotrophin secretion (Carter & Whitehead, 1981; Cheung, 1983) and in the development of Leydig cell

tumours (Bartke *et al.*, 1985). Both acute (Smith & Bartke, 1987) and chronic (Hodson *et al.*, 1980) hyperprolactinaemia suppressed basal LH secretion with minimal effects on FSH. Chronic hyperprolactinaemia, whether induced by ectopic pituitary transplants or chronic diethylstilbestrol treatment, was highly effective at reducing the incidence of Leydig cell tumours in Fischer 344 rats (Bartke *et al.*, 1985). However, Cimetidine, an H2-receptor blocking agent, which increase although inconsistently PRL levels (Fave *et al.*, 1977) increases the incidence of Leydig cell tumours. This effect was attributed to its anti-androgenic action (Funder & Mercer, 1979, Baba *et al.*, 1981). The effect of cimetidine on gonadotrophin levels is equivocal; Van Thiel *et al.* (1979) reported a decreased response to LHRH, whereas Vicburger *et al.* (1981) reported hypergonadotrophic hypogonadism during cimetidine treatment.

1.9. AIMS

A carcinogenic study by Sandoz revealed that treatment with the dopamine agonist (DA) (CU 32-085) for $2\frac{1}{2}$ years causes the development of Leydig cell tumours in rats. The aim of the present study was to investigate the changes in Leydig cell function *in vitro* at earlier stages of treatment *in vivo* with this compound which may be responsible for the formation of these Leydig cell tumours. Because it was possible that these changes took place in different populations of Leydig cells, methods for their separation, purification, and characterization were extensively investigated.

CHAPTER 2

MATERIALS and METHODS

2.1. MATERIALS

Type I Collagenase was obtained from Worthington Biochemical Corporation (Freehold, NJ, USA). Dulbecco's modified Eagle's medium (DMEM) (with 4500mg/l glucose and without pyruvate or $NaHCO_3$) was supplied either in 100ml concentrated solution (x10) or an equivalent amount (13.4g) in powdered form for 1l medium, and Earle's balanced salt solution (EBSS, x10) were purchased from Gibco Ltd, Uxbridge, Middlesex, UK). Dynabeads (M-450) coated with sheep anti-mouse IgG were obtained from Dynal (UK) Ltd, New Ferry, Wirral, Merseyside, UK. Mouse anti-rat macrophage antibody (ED2) from Serotec, Oxford, Oxon, UK. Ovine luteinizing hormone (oLH; batch oLH-26, potency: 2.3×10^3 units/g) and human chorionic gonadotrophin (hCG; batch CR127, potency: 14.9×10^6 units/g) were obtained from the National Institute of Child Health and Human Development (Bethesda, Maryland, USA). Crude hCG (5,000 IU) was obtained from Serono Laboratories (UK) Ltd, Welwyn Garden City, Hertfordshire. Carrier free $Na^{125}I$, ^3H-testosterone, L-[methyl-^3H] methionine (77Ci/ mmol), [1,2,6,7-^3H]-androst-4-ene-3,17-dione (80Ci/ mmol), ^3H_2O (5 mCi/ml), sheep anti-mouse Ig, horseradish peroxidase linked $F(ab')_2$ fragment were purchased from Amersham International plc, Amersham, Bucks, UK. [1ß-^3H]-androst-4-ene-3,17-dione (25.4Ci/ mmol) was obtained from New England Nuclear Corp. Bovine serum albumin (BSA, fraction V), soybean-trypsin inhibitor, Percoll, heparin (sodium salt from ovine intestinal mucosa), Diaminobenzidine-tetrachloride (DAB), adenosine 3':5' cyclic monophosphate

cAMP standard, succinyl cyclic AMP tyrosine methyl ester (ScAMP-TME), DMEM/ F12 base mixture (with 15mM Hepes without L-glutamine, L-leucine, L-lysine, L-methionine, $CaCl_2$, $MgCl_2$, $MgSO_4$, phenol red and sodium bicarbonate) supplied in a powdered form (14.8 g for 1l medium), ethylene glycol monoethyl ether, haematoxylin (HHS-1-16), dimethylpolysiloxane, 4-hydroxy-androstenedione (4OHA), (22R)-22-hydroxycholesterol, gelatin type B (from bovine skin), NAD^+, and NADH (disodium salt) were obtained from Sigma Chemical Company Ltd (Poole , Dorset, UK). Phosphate buffered saline (Dulbecco'A') tablets (code BR14a) was purchased from Unipath LTD, Hampshire, UK. Sep-Pak^R C18 cartridges were purchased from Waters Associates Inc and 3-Isobutyl-1-methylxanthine (IBMX) from Aldrich Chemicals co. PD-10 Sephadex G-25M columns, density marker beads for calibration of gradients of Percoll (Lot No NM 05514), and dextran T500 were obtained from Pharmacia, Uppsala, Sweden. Liquid scintillation cocktails, Ultima Gold and Ready Protein were obtained from Packard, (Ulgersmaweg, Groningen, The Netherlands) and Beckman (INC, Fullerton, CA) respectively. A polyclonal antiserum against 58 kDa inhibin, purified from bovine follicular fluid which was raised in a rabbit was a gift from Dr F.H. de Jong (Erasmus University, Rotterdam). Peroxidase conjugated goat-anti-rabbit immunoglobulin G antibody was obtained from Sanbio BV (Uden, The Netherlands). Glass microfibre filters (GF/C) and 31ET chromatography paper were purchased from Whatman Ltd (Maldstone, England). Bovine gamma globulin fraction II was obtained from ICN

Immunobiologicals, Lisle, IL. Technovit 7100 and Technovit 3040 were obtained from Kulzer et Co GnbH, Wherheim, FRG. Gill's haematoxylin (No3) was purchased from PolySciences, Warrington, PA, USA. Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad Laboratories GmbH, Heidemannstr München . Testosterone antibody (4R4 TR3), SU-10603-10603 (7-chloro-3, 4-dihydro-2-(3-pyridyl)-1-(2H)-naphthalenone), an inhibitor of 17α-hydroxylase activity and cyanoketone an inhibitor of 3ß-hydroxysteroid dehydrogenase activity were gifts from Dr F.F.G. Rommerts, Erasmus University, Rotterdam. Cyclic AMP antibody (MS1) was obtained from the Institute for Hormone Research, Hamburg, Germany. The other reagents were purchased from Sigma or BDH Ltd, Pooles, Dorset, UK. All solutions and buffers were made up in doubly glass-distilled water except where tap water was used.

2.2. TREATMENT OF ANIMALS

2.2.1. Dopamine agonist experiments

Age- and weight matched Sprague Dawley rats bred in our CBU unit were used. The animals were 8 weeks old at the start of the experiment, weighing about 180g. They were maintained on 41B powdered diet (Grain Harvesters Ltd) which contains 18.3% protein, 56.3% carbohydrate and 2.9% fat supplemented with vitamins and minerals. The diet was given at least one week before the start of treatment in order to determine the amount of food needed per animal. Drinking water was provided *ad libitum*. The animals, 2 to 5 per cage

were housed at 20-22°C with a 12h light, 12h dark cycle and were weighed twice per week. Mesulergine (CU32-085, DA) was given to the treated animals in food at a dose of 2mg/kg body weight/day for various periods of time. The control rats were given the same amount of powdered food. Food intake was measured before and during the treatment period in order to adjust the dose of DA given and also to determine whether the treatment has any effect on body weight. The rats were killed by anaesthesia with halothane followed by cervical dislocation. The testes were removed, weighed and kept in dissection media until used.

2.2.2. hCG and Dexamethasone Experiments

Male S.D. rats (250-300g) were randomly divided into 5 groups of 5 rats each and housed in the same conditions as described above. One group served as controls and the animals were given saline. The other groups were given 100 IU hCG, 100IU hCG + 1mg dexamethasone/kg body weight, and dexamethasone respectively. The vehicle, hCG, and dexamethasone were given s.c. in 100µl volume. Dexamethasone was always injected 1h before hCG as it was found not to have any effect when injected simultaneously with hCG. The rats were treated either once and killed 6h thereafter or treated once per day for 1 week and sacrified 24h after the last injection. The animals were killed and the testes were removed as described above.

2.3. Collection of testicular interstitial fluid

Collection of the interstitial fluid (IF) from individual testes was carried out using the

drip-collection technique, essentially the same procedure as described by Sharpe & Cooper (1983). Briefly, the testes were taken out, freed from surrounding tissue, placed into a preweighed tubes. A small incision was then made in the caudal end of each testicular capsule, the testes were replaced in the tubes, and the IF was allowed to percolate from the testis into the tube bottom for 20h at 4°C. Following centrifugation (1000 x g at 4°C for 5min) to precipitate any contaminating erythrocytes, the IF was aspirated and its volume measured. Although this method only recovers a proportion of the total IF present in the testis, the volume of IF recovered has been shown to provide a reliable index of the total testicular IF volume using either a radiolabel technique (Sharpe & Cooper, 1983) or morphometry (Widmark *et al.*, 1986).

2.4. Interstitial Cell Preparation

2.4.1. Collagenase digestion

Age- and weight-matched Sprague-Dawley rats weighing 300 to 500g were used in this study. Leydig cells were prepared essentially as described by Janszen *et al.*, 1976. Briefly, the testes from 5 or 10 control or DA-treated rats, or from rats treated with hCG, dexamethasone, or hCG + dexamethasone were decapsulated, without mechanically disrupting the testes. Two testes were then incubated in 7ml DMEM supplemented with with 10mmol Hepes/l, 0.1% BSA (w/v), 0.5g streptomycin/l, 250 x 10^3 units penicillin/l, 0.5g collagenase/l and 0.2mg soybean trypsin inhibitor/l. During the incubation, the cells were shaken longitudinally in a 30ml universal at a frequency of 70 oscillations/min at 37°C

until the interstitial tissue was completely dispersed (approximately 55min). after incubation, 15ml dissection medium was added to each tube. The tubes were inverted 40 times and the dispersed testes were allowed to settle. The cell suspension containing the Leydig cells, other interstitial cells, and some germ cells was then filtered through nylon mesh (60μm diameter) and sedimented by centrifugation at 220g for 10min.

2.4.2. Elutriation and Percoll Purification

Collagenase-dispersed interstitial cells from control and DA-treated rats were resuspended with elutriation buffer containing DMEM supplemented with 10mmol Hepes/l, 0.5g streptomycin/l, 250 x 10^3 units penicillin/l, 0.5% BSA (w/v), 1mmol EDTA/l, 31.8 x 10^3 units of heparin/l, adjusted to pH 7.4 with NaOH. The crude interstitial cells (600 to 800 x 10^6 cells) were loaded into the standard separation chamber of the elutriator (JE-6B, Beckman) at a flow rate of 19ml/min (using a Masterflex pump) and a constant rotor speed of 386 g. After the loading was completed, eight fractions were collected at different flow rates as shown in Table 2.1. Fractions of 50ml were collected at each step except the last flow rate (74 ml/min) at which 3 fractions of 50ml each were collected to ensure the complete elutriation of the remaining cells. After collecting the last fraction, the rotor was stopped without turning off the pump and a 50ml fraction was collected. This fraction contained very few intact cells and therefore was not considered.

Fraction number	Flow rate (ml/min)	Sedimentation velocity(mm/h ·g)
F1	19.6 ± 0.56	9.43 ± 0.29
F2	28.3 ± 1.15	13.6 ± 0.52
F3	36.3 ± 1.15	17.4 ± 0.52
F4	44.3 ± 1.15	21.3 ± 0.52
F5	52.0 ± 1.00	24.9 ± 0.40
F6	60.3 ± 2.08	28.9 ± 0.94
F7	68.3 ± 2.08	32.8 ± 0.93
F8	76.3 ± 2.08	36.6 ± 0.87

Table 2.1. Sedimentation velocities of elutriated testicular interstitial cells. The sedimentation velocities were determined as described by Grabske *et al.* (1975) using the equation: $S= 1.93 \times f/R^2$, where S is the sedimentation velocity of the cell (mm/h·g), f is the volumetric flow rate (ml/min), R is the rotor speed expressed in units of 10^3 rpm and 1.93 is a constant factor involving chamber and rotor characteristics and conversion from minutes to hours and centimeters to millimeters. Results are presented as mean ± SD of 3 experiments.

The cells from each of the eight fractions (F1 to F8) were sedimented by centrifugation at 220g for 10min, resuspended in 2ml DMEM and carefully added to the top of a 0-90% linear Percoll gradient. The gradients were centrifuged at 1300g for 25min. The cells from the Leydig cell rich band (band 2 from the top of the gradient) were isolated by removal with a Pasteur pipette from the top of the gradient. The cell suspensions were diluted with three volumes of dissection medium, centrifuged at 360g for 5min and resuspended in an appropriate volume of DMEM.

Aliquots from cell suspension from each fraction (before and after Percoll purification) were characterized for macrophages and 3ß-hydroxysteroid dehydrogenase isomerase (3ß-HSD). Only bands rich in Leydig cells (band 2) from fraction F3 to F8 were subjected to further assays.

In freshly isolated cells, cell numbers were determined in each fraction as the mean of four different fields each containing at least 400 total cells using a haemocytometer. The Leydig cells were identified by their distinctive yellow halo under phase contrast microscopy and there was no significant difference between this parameter and the presence of 3ß-HSD determined by cytochemistry (Table A1.2.1). Cell handling and transfer were kept to a minimum throughout all procedures to reduce the risk of cell damage or loss.

2.4.3. Isolation of Macrophages Using Dynabeads (M-450) Coated With ED2

On the basis of preliminary experiments showing that macrophages are mostly eluted in the first 3 fractions (F1 to F3) and only few are present in later

fractions F4 to F8, two fractions (100ml and 150ml) were collected at flow rates of 35 and 76ml/min respectively (corresponding to F1-F3 and F4-F8 respectively). Both fractions were then separately purified on Percoll gradients and the band rich in Leydig cell was collected. The purified first fraction contained 9.6 ± 1.51 % Leydig cells (average density 1.045 g/ml) (0.6 ± 0.112 x 10^6 Leydig cells/ testis) contaminated with germ cells, macrophages, and other unidentified cells and was therefore not investigated further (results represent combined data from two different experiments and presented as mean ± SD of 8 different fields of at least 500 total cells each). The second fraction contained 96.2 ± 2.06 % Leydig cells (average density 1.075 g/ml) (2.5 ± 0.79 x 10^6 Leydig cells/ testis) (results presented as mean ± SD of ten different experiments). The latter, which is used routinely in our laboratory for studies on Leydig cells was further purified with Dynabeads (M-450) coated with a macrophage monoclonal antibody. The Dynabeads (M450) pre-coated with the sheep anti-mouse IgG were incubated overnight at 4°C with the macrophage antibody (ED2) at a concentration of 2μg/mg Dynabeads with continuous mixing with a rotating mixer. The coated Dynabeads were isolated magnetically using a Dynal magnetic particle concentrator (MPC). They were washed four times with phosphate buffered saline (PBS; pH 7.4) containing 0.1% BSA (w/v) with continuous mixing for 30min for each wash. The Dynabeads were isolated magnetically between each wash. The antibody-coated Dynabeads were kept at 4°C in PBS containing 0.1% BSA and 0.02% sodium

azide (w/v) and used within 2 weeks. They were washed twice before use as described above to remove sodium azide. The elutriated-/ Percoll-purified testicular interstitial cells were incubated with the antibody-coated Dynabeads (5×10^6 cells per 4×10^7 Dynabeads) for 30min at room temperature (RT) or on ice with mixing every 2 to 5 min and then separated magnetically. This process was repeated to remove any residual free beads and/or macrophages. The cells obtained were analysed for macrophage content and testosterone production and compared with the purified cells that had not been subjected to further purification with Dynabeads.

2.5. Determination of Leydig cell volume

The cell volume was determined using a Coulter Counter^R (model Z_{B1}) and Channelyzer^R (Coulter Electronics Limited, Luton, Beds, UK) which were calibrated and checked with reference particles (14.5 µm diameter). The elutriated and Percoll-purified Leydig cells were diluted (1:4000) with Isoton II (azide free balanced electrolyte solution) then counted at an amplification= 16, aperture current= 0.50, and count range= 400.

2.6. 3ß-HSD and Diaphorase Cytochemistry

Cytochemical staining for 3ß-hydroxysteroid dehydrogenase isomerase (3ßHSD) and diaphorase (viability) were determined as described by Cooke *et al.*, 1983. For 3ß-HSD cytochemistry, an equal volume of 6% dextran (w/v) was added to the cell suspension which was then frozen at -20°C for at least 1h up to 1 week to permeabilize the cells. When assayed, the cells were first

sedimented by centrifugation at 500 g for 3min, then resuspended in a reaction mixture (500µl/ 0.5 x 10^6 cells) containing 5α-androstene 3ß-ol-17-one, nitroblue tetrazolium, nicotinamide and NAD^+ in 0.1M phosphate buffer (pH 7.1-7.3) and incubated for 1h at 37°C (see Appendix 1.2.1 for details). 5α-Androstan 3ß-ol-17-one was omitted from the control samples. After incubation, the cells were sedimented by centrifugation at 500 g for 3min and washed once, resuspended in an appropriate volume of DMEM (0.5 x 10^6 per 500µl), and centrifuged at 90 g for 5min onto glass microscope slides (100µl/ slide) in a cytocentrifuge (cytospin 2) (Shandon, Southern Products, Cheshire, UK). The cells were then fixed for 30min in 10% formaldehyde/ 50% ethanol (v/v), allowed to dry, then mounted in aqueous mounting medium or gelatin solution. The percentage of positively stained cells was estimated by counting a minimum of 5 fields of about 500 cells each under the light microscope.

Cytochemical staining for diaphorase was performed by a similar method. The reaction mixture contains nitroblue tetrazolium and NADH in 0.1M PBS (pH 7.1-7.3) (see Appendix 1.2.2 for details). NADH was omitted from the control samples. The cells tested for the viability were incubated in 24 well plates under the same conditions as those used for measuring the LH or other ligands response (see below) before performing the assay. When assayed, the incubation medium was removed carefully with a Pasteur pipette and the reaction mixture was added (500µl/10^5 cells/well). After incubation for 1h, the reaction mixture was removed and the cells were fixed for 30min in 10% formaldehyde/ 50%

ethanol (v/v). The percentage of positively stained cells (non viable) was determined as described above for 3ß-HSD.

2.7. Macrophage Immunocytochemistry

Macrophages were characterized using an immunoperoxidase staining technique as described by Dijkstra *et al.* (1985). Testis interstitial cells were diluted in DMEM (0.5×10^6 cells/ml) and centrifuged at 90 g for 5min onto glass microscope slides (100µl/ slide) using a cytocentrifuge. The cell preparations were fixed in acetone/chloroform (1:1, v/v) for 10min, air dried, and then kept at -20°C until assayed. All incubations were carried out at room temperature in a humid chamber. Endogenous peroxidase was inhibited by incubating the fixed cells with 0.1% hydrogen peroxide (H_2O_2, v/v) or 0.25% periodic acid (v/v) in 0.01 mol PBS/l (pH 7.4) for 10min. The cells were then washed with 0.01 M PBS pH 7.4 and incubated with a rat macrophage monoclonal antibody (ED2) at a dilution of 1:500 in PBS for 2h. After washing in 0.01 M PBS (pH 7.4) for 15min, the sheep anti-mouse Ig horseradish peroxidase linked $F(ab')_2$ fragment was added at a dilution of 1:100 and incubated for 1h. After washing in PBS, the slides were stained for peroxidase activity by incubating the cells with DAB (0.1g DAB in 10 mmol Tris/HCl buffer/l, pH 7.6 containing 0.01% H_2O_2) for 15min. Control slides were incubated in the same way omitting the first antibody (ED2). Finally, the cells were washed with PBS three times, counterstained with haematoxylin, dehydrated, and mounted in DePeX mounting medium. The percentage of

macrophages was evaluated by counting at least five fields (n=5) of about 500 cells each using a light microscope.

2.8. Leydig Cell Incubations

The purified Leydig cells were plated out in Costar 24 well plates at a density of 10^5 (response to LH), 1.5 x 10^5 (^{125}I-labelled hCG binding), 4 x 10^5 (aromatase activity) cells per well or in Costar 6 well plates at a density of 10^6 (Scatchard analysis) and 2 x 10^6 cells per well (protein synthesis). The cells were preincubated in an air incubator for 2h at 34°C in 1ml DMEM pH 7.4 containing 0.1% BSA, 0.5g streptomycin/l, 250 x 10^3 units penicillin/l and 10mmol Hepes/l or in 5ml DMEM/ F12 pH 7.4 containing 10mol methionine/l (protein synthesis). The cells were then incubated as described below.

2.9. Binding of ^{125}I-labelled hCG to Leydig cells

^{125}I-labelled hCG binding was performed essentialy as described by Clausen *et al.* (1981) Human chorionic gonadotrophin (hCG, CR127) was iodinated by the lactoperoxidase method and purified by sephadex G-25M chromatography using PD-10 columns (Thorell & Johansson, 1971). The specific activity of ^{125}I-labelled hCG was 30-50 µCi/µg at the time of preparation. The ^{125}I-labelled hCG was used within 3 to 4 weeks of preparation.

After preincubation of the cells, the media was changed and ^{125}I-labelled hCG (0.56 nmol/l) was added in the presence of 0.14 nmol of unlabelled hCG/l. Non-specific binding (NSB, <5% of total radioactivity in the incubation mixture) was determined in the presence of 400 IU of unlabelled crude hCG. The cells

were then incubated overnight at 4°C. Scatchard analysis of specific binding of ^{125}I-labelled hCG to Leydig cells was determined by incubating purified Leydig cells (10^6 Leydig cells per well in 6 Costar well plates) with increasing concentrations of ^{125}I-labelled hCG (0.55 to 8.3 nmol hCG/l) in the presence (to determine non-specific binding) or absence of 27nmol unlabelled hCG/l overnight at 4°C. The level of binding was determined by aspirating the medium, washing twice with 500µl DMEM to remove unbound hormone and dissolving the cells in 500 µl 0.5mol NaOH/l. The bound radioactivity was measured in an LKB 1275 gamma counter (Wallac OY, Turku, Finland).

^{125}I-labelled hCG binding in crude testicular interstitial cells was determined by incubating 1/4 of interstitial cells obtained from each testis in LP4 tubes in 1ml DMEM containing 0.1% methyl cellulose. The cells were preincubated for 2h at 34°C in a shaking water bath (70 strokes/ min). ^{125}I-labelled hCG (0.56 nmol/l) was then added in the presence of 0.14 nmol unlabelled hCG/l and incubated overnight at 4°C. The reaction was stopped by placing the tubes on ice for 15min and then the cells were washed twice with 2ml ice-cold DMEM and the cell pellet was collected by centrifugation at 1000 g for 10min. The non-specific binding for each testis from an individual animal was determined by adding 400IU of unlabeled crude hCG. The cell pellet was dissolved in 500µl 0.5mol NaOH/l and counted in a gamma counter.

2.10. Testosterone, Pregnenolone and Cyclic AMP Assays

The preincubated cells were incubated for 2h in the absence or presence of

oLH or other ligands (dcAMP/l and 22R-hydroxycholesterol/l). In the experiments where dexamethasone was used *in vitro*, this was added at a concentration of 25µmol/l to the preincubated cells 30min prior to the addition of LH or dcAMP. In the experiments in which pregnenolone was measured, inhibitors of pregnenolone metabolism were added 30min before adding the ligands at a concentation of 5µmol and 20µmol/l for cyanoketone and SU-10603-10603 respectively. In the experiment showing the *in vitro* effects of DA, DA was added at different concentrations (10^{-8} to 10^{-5}mol/l) 2h before the addition of 3.3nmol LH/l or 1mmol dcAMP/l.

Testosterone, pregnenolone, and cAMP were extracted from the cells and media by the addition of $HClO_4$ (final concentration 0.18mol/l) which was neutralized by adding K_3PO_4 (final concentration 0.19mol/l). The extracts were stored at -20°C and testosterone (Verjans *et al.*, 1973), pregnenolone (van der Vusse *et al.*, 1975), and cAMP (Steiner *et al.*, 1972; Brooker *et al.*, 1979; Delaage *et al.* 1979) production was determined by radioimmunoassay. The antiserum against pregnenolone cross-reacts less than 5% with dehydroepiandrosterone, testosterone, 5α-dihydrotestosterone, 17-hydroxyprogesterone, 17-hydroxypregnenolone, 20-dihydropregnenolone; 12% with progesterone and 59% with 5α-pregnanolone (Haren *et al.*, 1989). Iodination of ScAMP-TME was performed as described by Brooker *et al.* (1979), or Wilson (1988).

2.11. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) Assay

Cells from controls and rats treated with hCG, dexamethasone (Dex), or hCG + Dex were incubated in 24 well plates (4 x 10^5 cells/ well) for 3h. $PGF_{2\alpha}$ was extracted from cells and media by the addition of $HClO_4$ which was neutralized with K_3PO_4. The extracts were stored at -20°C and $PGF_{2\alpha}$ was measured by radioimmunoassay in Prof. A.P. Flint laboratory (Institute of Zoology, London) as described by Kelly *et al.* (1986) using the antiserum provided by Dr R. Kelly (MRC Reproductive Biology Unit, Edinburgh).

2.12. Aromatase Assay

Aromatase activity was determined by measuring ^3H_2O resulting from the aromatization of [1,2,6,7-^{3}H]androstenedione as described by Pasanen (1985) with some modifications. Briefly, elutriated/ Percoll purified Leydig cells (4 x 10^5 cells per well) from control and DA-treated rats were preincubated for 2h in 1ml DMEM. The media was changed and the cells were incubated with or without an inhibitor of aromatase activity, 4hydroxyandrostenedione (4OHA) (100µmol/l), for 30min. The substrate, [1,2,6,7-^{3}H]-androstendione (0.2 µCi) was then added and the cells were incubated for 2h at 34°C in an air incubator. The reaction was stopped by adding 100µl ice-cold 33.3% TCA (w/v). The non-specific activity was determined by the addition of 100µl TCA before adding the substrate. In some experiments ^3H_2O (1µCi) was added at the start or at the end of the incubation period to assess the possible loss of ^3H_2O resulting from aromatization by evaporation during incubation. The incubation media was

transferred into 1.5ml Eppendorf tubes and centrifuged for 3min at 8000 g. The supernatant (1ml) was taken into a 1ml plastic injection syringe and pushed through a pre-equilibrated Sep-Pak^R minicolumn into a counting vial. The column was then washed with 2ml water to remove all ^3H_2O. Ten ml of liquid scintillation cocktail (Ultima Gold) was added and the radioactivity was counted using an LS 5000 CE liquid scintillation counter (Beckman Instruments, INC., Fullerton, CA). The columns were pre-equilibrated or regenerated by washing successively with 10ml water, 5ml ethanol and 10ml water.

2.13. Protein Synthesis

This was measured by the incorporation of ^{3}H-methionine into proteins. Elutriated/ Percoll-purified Leydig cells from control and DA-treated rats were incubated at a density of 2 x 10^6 cells per well in 6 Costar well plates. The cells were preincubated in DMEM/ F12 containing 10mM unlabelled methionine for 2h. The cells were then washed twice with 1ml methionine free media and recultured in 5ml methionine free medium to which 5µCi ^{3}H-methionine was added. Inhibitors of protein synthesis, actinomycin D (transcriptional inhibitor) (5µg/ml) and cycloheximide (translational inhibitor) (10µg/ml) were added 30min before ^{3}H-methionine was added. After incubation with L-[methyl-^{3}H]-methionine for 4h, the cells were washed twice with 5ml medium containing 10mmol unlabelled methionine/l. The cells were then scraped and homogenized in 1ml PBS pH 7.4 containing 1% BSA and 10mmol methionine/l. Three aliquots from each homogenate were placed on Whatman

glass microfibre filters (GF/C, 2.5cm diameter) prewashed with PBS/ BSA/ methionine. The protein was precipitated under vacuum by adding 3 x 1ml 10% TCA containing 10mmol unlabelled methionine/l. The filter was then put in a scintillation vial and 10ml liquid scintillation cocktail (Ready Protein) was added and counted in a Beckman ß-scintillation counter. Protein content was determined by the Bio-rad protein assay as described by Bradford (1976).

2.14. Histological and immunohistological Procedures

Testes were fixed in Bouins solution for 48h. All testes were cut in half after the first 12h fixation. After dehydration, the material was embedded in Technovit 7100 plastic, a glycol methacrylate (Kulzer and Co GmbH, Wehrheim, F.R.G.), or in paraffin for immunohistochemical purposes. Five µm sections were cut, at least 1mm away from the edge where the testis was cut in half, in order to avoid damage to the interstitial compartment. The plastic sections were stained by the periodic acid Schiff (PAS) technique and Gill's haematoxylin (Polysciences Inc., Warrington, PA, USA).

The immunohistochemical localization of inhibin-like material was carried out as described by Teerds *et al.* (1991). Briefly, 5µm thick paraffin embedded sections were used. Sections were deparaffinized and endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 30min. The slides were subsequently washed in 0.01M PBS (pH 7.4). This was followed by preincubation with 10% normal goat serum in PBS for 30min. The slides were then incubated at room temperature with either the inhibin antibody diluted

1:200 in PBS with 0.2% Tween 20 for 60 to 120min, with 1% normal rabbit serum, or with 1% bovine serum in PBS with 0.2% Tween 20. Following this incubation, the slides were rinsed with PBS and then incubated for 45 min with peroxidase conjugated goat anti-rabbit immunoglobulin G in PBS with 0.2% Tween 20. Slides were again washed in PBS and bound antibody was visualized with a 0.5 mg/ml solution of DAB in 0.05M Tris/HCl (pH 7.6), 0.1% hydrogen peroxide for 4 min. No non-specific staining with rabbit or bovine serum could be detected, indicating that the inhibin antibody bound specifically. The specificity of the second antibody (goat anti-rabbit immunoglobuline G) was checked by omitting the first antibody incubation step. Non-specific staining with the second antibody was not detected. The slides were counterstained with haematoxylin.

2.15. Cell Counts

Identification of Leydig cells and macrophages was based on their nuclear morphology and staining characteristics of their cytoplasm according to de Kretser & Kerr (1988) and Hardy *et al.* (1989). Nuclei of Leydig cells, macrophages and Sertoli cells were counted. Leydig cells were recognized by their spherical to oval nucleus with a characteristic distribution of heterochromatin and blue/ purple-staining cytoplasm. Macrophages were identified by their often irregularly shaped and somewhat smaller nucleus and pink (PAS-positive) cytoplasm. Cross-sections through the whole testis were made in which areas were chosen at random. Nuclei of cells in these areas

were counted using a square lattice grid inserted in the eyepiece of the microscope. At least 5 different sections, 100-200μm apart were counted until 1000 Sertoli cells were scored. The number of nuclei counted per cell type (henceforth called number of cells) was expressed per 1000 Sertoli cell nuclei according to the method of Heller *et al.* (1971).

Since it has been demonstrated that the number of Sertoli cells per testis in the intact rat does not undergo changes after day 15 of postnatal life (Bortolussi *et al.*, 1990), it was not necessary to apply corrections for changes in the number of Sertoli cells. In addition, it has been reported that hypophysectomy or treatment with gonadotrophins has no effect on Sertoli cell number (Steinberger, 1973).

2.16 Analysis of data

The concentrations of testosterone, pregnenolone, and cAMP in the analysed samples were determined using a radioimmunoassay DATA analysis programme (IPM-PC Version 1.1 by KC Peley, 1978). Scatchard analysis of the binding of ^{125}I-labelled hCG to Leydig cells was carried out using the EBDA radioligand binding analysis programme by GA McPherson (Elsevier-BIOSOFT).

Statistical analysis was carried out using a Minitab DATA analysis program (Minitab, INc, PA, USA). The significance of differences was assessed by one way analysis of variance or Student *t*-test. A p value less than 0.05 was considered statistically significant. Data are presented as means ± (SD) and n is the number of experiments.

CHAPTER 3

SEPARATION AND CHARACTERIZATION OF LEYDIG CELLS AND MACROPHAGES FROM RAT TESTES

3.1. SUMMARY

A method involving centrifugal elutriation followed by density gradient centrifugation and incubation with a macrophage monoclonal antibody, has been investigated to separate and characterize Leydig cells and macrophages from adult rat testes.

After dispersion of the testes with collagenase, the isolated interstitial cells were found to contain 18% Leydig cells and 12% macrophages. These cells were then separated by centrifugal elutriation into eight fractions (F1 - F8) (9.6 to 76.3 ml/min, at 386 g). Each of these fractions was then further purified by density gradient centrifugation on 0-90% Percoll gradients. After centrifugal elutriation, the macrophages were mainly eluted in the first three fractions (F1 - F3), whereas the Leydig cell percentage increased in each fraction with increasing flow rate. After further purification of each fraction on Percoll gradients, high percentages of macrophages (11-20%) were found in fractions F1 - F3 (average density 1.045 g/ml), containing 11-37% Leydig cells. Less than 3% of the cells in fraction F4-F8 (average density 1.075 g/ml) were macrophages and more than 95% were Leydig cells.

Heterogeneity of Leydig cells with respect to sedimentation velocities, cell volume and function was found. Leydig cells from elutriated/ Percoll-purified fractions F4 - F8 were heterogeneous with respect to cell volume, testosterone and cAMP production but showed a similar binding capacity for ^{125}I-labelled human chorionic gonadotrophin. Leydig cells with the highest sedimentation velocity and cell volume from fractions F7 and F8 were approx. 2 fold more

responsive to LH (3.3 nmol/l) with respect to testosterone and cAMP production compared with Leydig cells with the lowest sedimentation velocity and cell volume.

The elutriated/ Percoll-purified cells (corresponding to fractions F4 - F8) were further purified by incubation with magnetic beads coated with a macrophage monoclonal antibody; this yielded very pure Leydig cells containing <0.3% macrophages. The incubation temperature (room temperature or 4°C) during the purification with magnetic beads did not affect the degree of purity or the responsiveness of the Leydig cells to LH. The removal of the remaining macrophages with magnetic beads did not have any significant effect on the Leydig cell responsiveness to LH. Furthermore, addition of macrophages enriched testicular interstitial cells to elutriated/Percoll-purified Leydig cells had no significant effect on their responsiveness to LH.

It is concluded that Leydig cells purified by elutriation and density gradient centrifugation are heterogeneous with respect to their sedimentation velocities, cell volume, and responses to LH; the higher the sedimentation velocity, the higher is their capacity to respond to LH. Leydig cells free from macrophages can be prepared by further purification using magnetic beads coated with a macrophage monoclonal antibody.

3.2. INTRODUCTION

Many workers routinely purify Leydig cells from rat and mouse testes by density gradient centrifugation to obtain a purity of 60-85% using Ficoll (Janszen *et al.*, 1976; Conn *et al.*, 1977; Molenaar *et al.*, 1983), Metrizamide (Conn *et al.*, 1977; Payne *et al.*, 1980; O'Shaughnessy *et al.*, 1981; Chase & Payne, 1983), and Percoll (Schumacher *et al.*, 1978; Cooke, *et al.*, 1981; Aldred & Cooke, 1982; Gale *et al.*, 1982; Aldred & Cooke, 1983; Browning *et al.*, 1983; Laws *et al.*, 1985; Hedger & Eddy, 1986; Risbridger & de Kretser, 1986; Hedger & Eddy, 1987; Browne *et al.*, 1990). In order to obtain a higher degree of purity (>95%), a combination of centrifugal elutriation and density gradient centrifugation has been used (Aquilano & Dufau, 1984; Klinfelter *et al.*, 1987; Platts *et al.*, 1988).

In many of the studies using density gradient centrifugation, heterogeneity of the Leydig cells has been reported. However, it has not been resolved as to whether this is a result of cell damage caused by the isolation procedures used or truely reflects functional differences. Also little attention has been paid to the influence of the other cell types present in these preparations on Leydig cell function. For example macrophages, which are a major contaminant of density gradient fractions containing Leydig cells (Laws *et al.*, 1985; Hedger & Eddy, 1986; Risbridger & de Kretser, 1986), have been shown to secrete factors which may modulate Leydig cell function (Yee & Hutson, 1985, Lin *et al.*, 1987; Bellvé & Zheng, 1989; Calkins *et al.*, 1990). This study was therefore carried out to investigate further the heterogeneity of Leydig cells in highly

purified preparations using centrifugal elutriation and density gradient centrifugation. We have also developed a method to produce macrophage-free Leydig cell preparations using magnetic beads coated with a macrophage monoclonal antibody.

3.3. RESULTS

3.3.1. Leydig cell content

The crude testis interstitial cell suspension, which contained 17.6 ± 0.65 % Leydig cells (4.46 x 10^6 cells per testis) (Table 3.1), was separated by elutriation into eight fractions (F1 to F8) by varying the flow rate from 19 (± 0.56) to 76.3 (± 2.08) ml/min (this corresponded to sedimentation velocities of 9.4 (± 0.29) to 36.6 (± 0.87) mm/h·g) as shown in Table 2.1 (see chapter 2). Fractions F3 to F8 contained a high percentage of Leydig cells (36-66%), while only 13 and 17% Leydig cells were found in fractions F1 and F2 respectively (Figure 3.1a). After purification of each fraction on Percoll gradients, two bands were formed (band 1 and 2). Band 1 (at the top of the gradient) was not investigated further because it contained very few Leydig cells contaminated with cell debris, together with other cells including macrophages and germ cells. Band 2 (average density 1.045 g/ml) of fractions F1 to F3 contained 11 - 37% Leydig cells contaminated with red blood cells, macrophages, germ cells and other unidentified cells, while band 2 (average density 1.075 g/ml) of fractions F4 to F8 contained more than 95% Leydig cells (Figure 3.1b). Because of the relatively low percentage of Leydig cells in fraction F1-F3, these fractions were not subjected to further assays.

3.3.2. Macrophage Content

The crude testis interstitial cell suspension contained 11.5 ± 3.25 % macrophages (3.36 x 10^6 cells per testis) (Table 3.1.). After centrifugal elutriation, the macrophages were mainly found in fractions F1 to F3 (10 - 18%) while the later fractions F4 to F8 contained 2-5% macrophages (Figure 3.1a). After purification on Percoll gradients, a relatively high percentage of macrophages (11 - 20%) was found in band 2 (1.045 g/ml) of fractions F1 to F3 (Figure 3.1b). In contrast band 2 (1.075 g/ml) of fraction F4 to F8 contained 1.5 - 2% macrophages (Figure 3.1b). The latter was further purified using Dynabeads coated with a macrophage monoclonal antibody (ED2) to remove the remaining macrophages. This procedure yielded very pure Leydig cells containing 0.23 ± 1.50 and 0.09 ± 0.04 % macrophages at room temperature and 4°C respectively (Table 3.2).

3.3.3. Leydig cell volume

As shown in Figure 3.2., the cell volume was significantly higher ($P < 0.05$) in Leydig cell fractions F6 to F8 compared with that in fractions F1 to F5. This suggests the presence of at least two populations of Leydig cells with different cell volume.

Cell type	Percentage (%)	Number per testis ($x10^{-6}$)
Leydig cells	17.6 ± 0.65	4.46 ± 0.65
Macrophages	11.5 ± 3.25	3.36 ± 0.56

Table 3.1. Leydig cell and macrophage content in crude testicular interstitial cells. The Leydig cells were identified by cytochemical staining for 3ß-hydroxysteroid dehydrogenase isomerase (3ß-HSD) and the macrophages were characterized by immunocytochemistry using a macrophage monoclonal antibody ED2. The number and percentage of Leydig cells and macrophages were determined by counting at least 5 fields of 500 cells each. Results are presented as means ± SD of 4 (Leydig cells) and 15 (macrophages) separate experiments, each performed in triplicate.

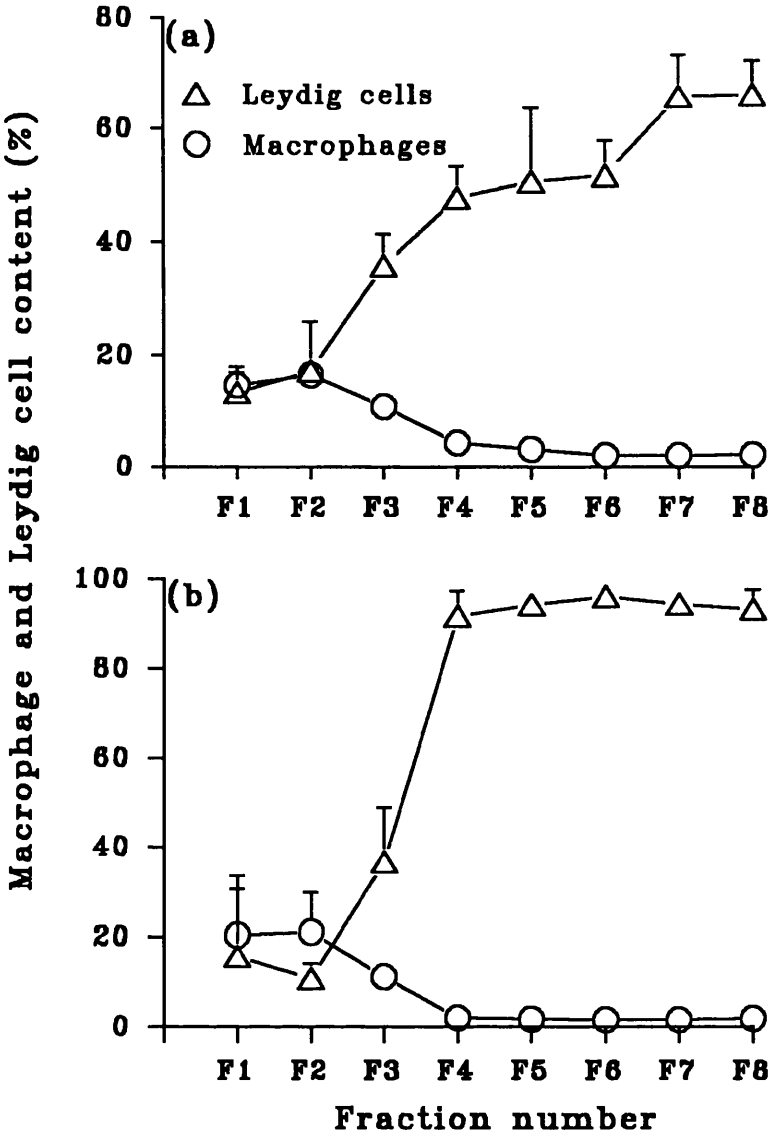


Figure 3.1. Macrophage and Leydig cell content in elutriated (a) and elutriated/ Percoll-purified (b) interstitial cell fractions from rat testes. Aliquots from elutriated fractions (F1 to F8) and from the same fractions after purification on Percoll were assayed for Leydig cells and macrophages. The Leydig cells were identified by cytochemical staining for 3ß-HSD, and the macrophages were characterized by immunocytochemistry using a macrophage monoclonal antibody ED2. The percentage of Leydig cells and macrophages within each fraction were determined by counting at least 5 fields of 500 cells each. Results are presented as means ± SD of four separate experiments, each performed in triplicate.

Fraction	Macrophage content (%)
Elutriated	5.1 ± 1.50
Elutriated + Percoll	2.1 ± 1.39
Elutriated + Percoll + Dynabeads at RT	0.23 ± 0.08
Elutriated + Percoll + Dynabeads at 4°C	0.09 ± 0.04

Table 3.2. Macrophage content in testicular interstitial cells after elutriation, purification on Percoll gradients, and a further purification with Dynabeads coated with ED2. The percentage of macrophages was determined as described in Table 3.2. Aliquots from the fraction corresponding to fractions F4 to F8 after elutriation; elutriation and purification on Percoll; elutriated, purified on Percoll, and incubated with Dynabeads coated with a macrophage monoclonal antibody ED2 (at room temperature (RT) or at 4°C). Results are presented as means ± SD of 4 separate experiments performed in triplicate.

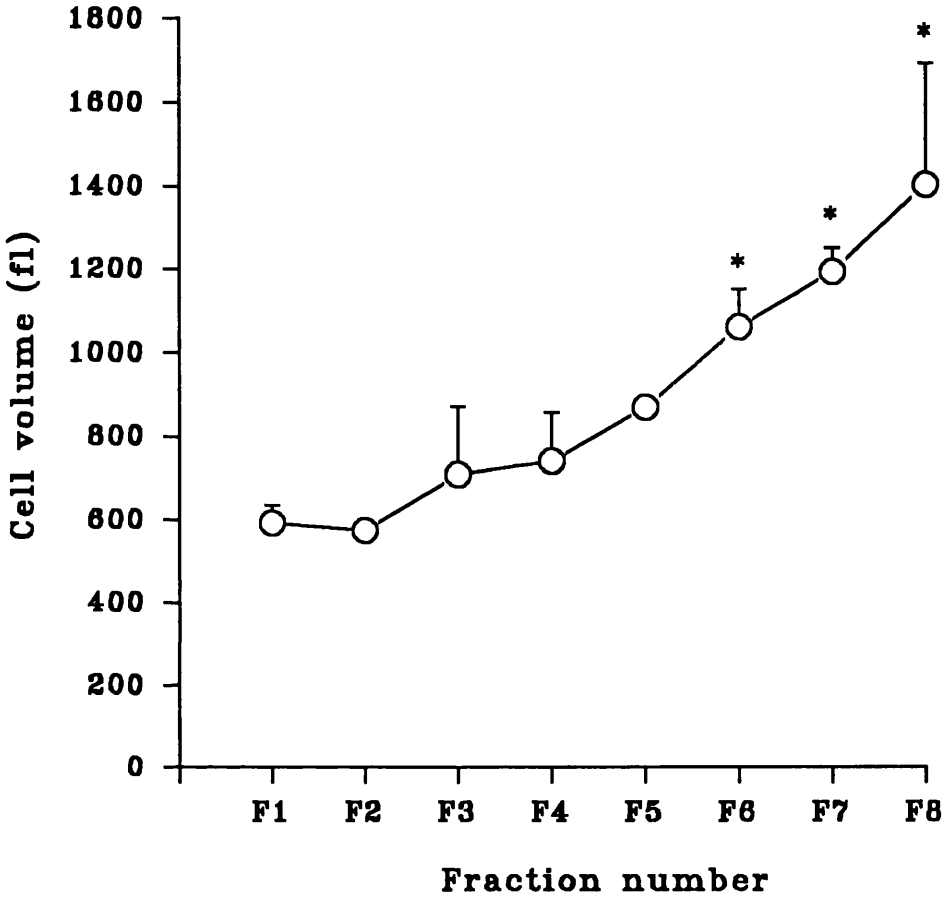


Figure 3.2. Leydig cell volume in elutriated/Percoll-purified testicular interstitial cell fractions. Aliquots from elutriated/Percoll-purified interstitial cell fractions were analysed for cell volume using a coulter counter. Results are presented as pooled data from two separate experiments each performed in triplicate. *P<0.05 F7 and F8 compared with F1, F2, F3, F4 and F5, and F6 compared with F1 and F2.

3.3.4. Specific binding of ^{125}I-labelled hCG to Leydig cells and testosterone and cAMP production

Although no significant difference was observed in the specific binding capacity to ^{125}I-labelled hCG of the Leydig cells from all the fractions (F4 - F8; 38 ± 9.7 fmol/10^6 Leydig cells) (Figure 3.3a), cells from fractions F7 and F8 produced higher levels of testosterone in response to LH compared with cells from fraction F4 or F5 ($P<0.05$) (testosterone production in cells from fraction F8 in response to LH was 610 ± 177 pmol/ 10^6 Leydig cells/ 2h) (Figure 3.3b). Production of cAMP was significantly ($P<0.05$) higher in cells from fractions F7 and F8 compared with cells from fractions F4, F5 or F6 (cAMP production in cells from fraction F8 in response to LH was 280 ± 77.7 pmol/ 10^6 Leydig cells/ 2h) (Figure 3.3c). The basal levels of testosterone (35 ± 17.6 pmol/10^6 Leydig cells/2h) and cAMP (7.02 ± 1.55 pmol/10^6 Leydig cells/2h) production were similar in cells from all the fractions (F4 to F8). The difference between fractions with respect to testosterone and cAMP production is not due to a difference in Leydig cell content (Figure 3.1b) and/or cell viability; the mean cell viability for the five fractions (F4 to F8) was 97 ± 1.3 (n=3 different experiments) and the results are calculated and expressed per viable Leydig cell.

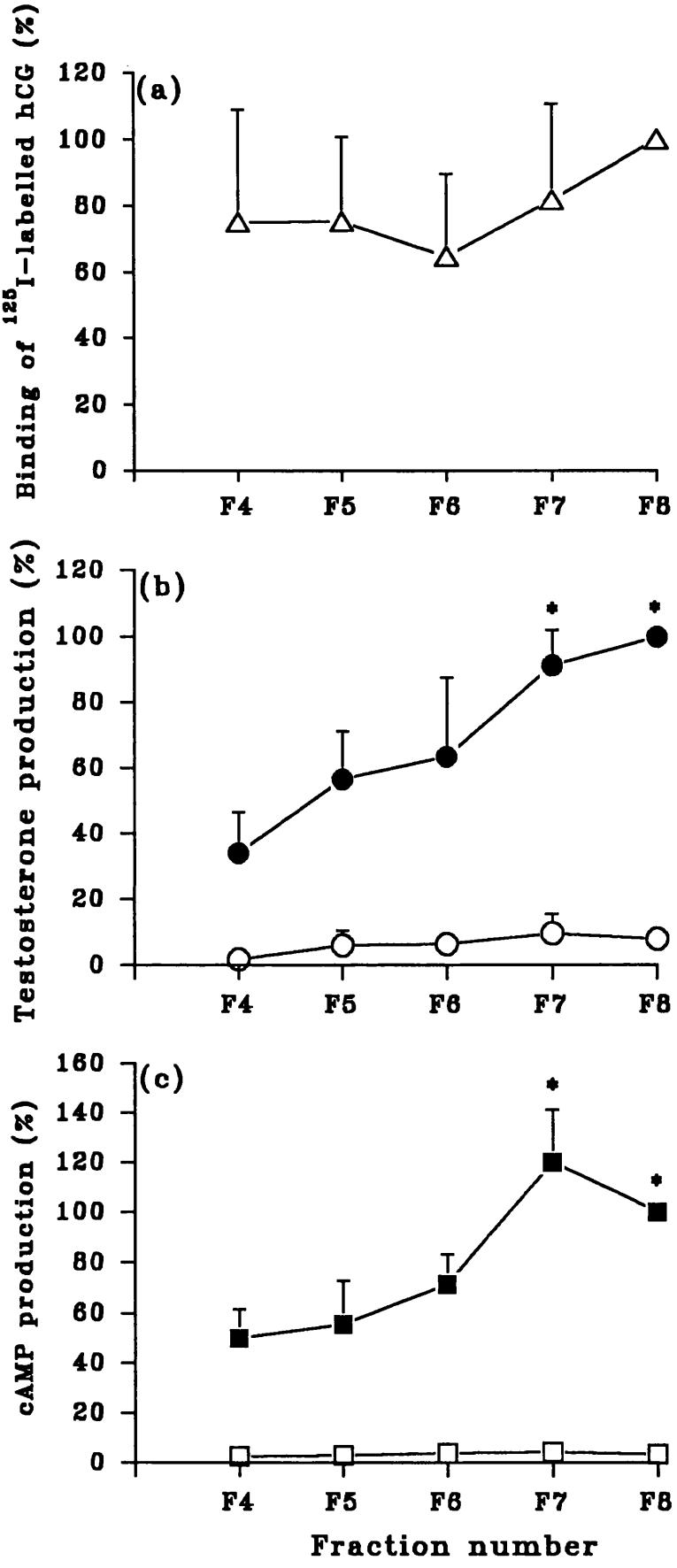
After purification with Dynabeads, the responsiveness of the Leydig cells to LH (0.033nmol/l and 3.3nmol/l) with respect to testosterone (Figure 3.4a) and cyclic AMP (Figure 3.4b) production was not significantly different from that of the cells not subjected to Dynabeads. Purification with Dynabeads did not

have any effect on cell viability (Table 3.3). The incubation temperature (room temperature or 4°C) during purification with Dynabeads did not affect the degree of purity of Leydig cells and their responsiveness to LH with respect to testosterone and cAMP production.

Addition of macrophage enriched testicular interstitial cells to elutriated/ Percoll-purified Leydig cells (the macrophage content was 10.4%) had no effect on basal or LH-stimulated testosterone production by the purified Leydig cells (Table 3.4).

Figure 3.3. Specific binding of ^{125}I-labelled hCG to Leydig cells (a) and production of testosterone (b) and cAMP (c) in elutriated/ Percoll-purified interstitial cell fractions from rat testes.

Cells from fractions F4 to F8 were plated out in Coster 24 well-plates at a density of 1.5 x 10^8 (^{125}I-labelled hCG binding) and 10^8 (response to LH) cells/l and preincubated for 2h in an air incubator at 34°C. Binding of ^{125}I-labelled hCG to Leydig cells (open triangles) was carried out at 4°C and non-specific binding values (<5% of the total radiactivity added to the incubation mixture) have been substracted. Production of testosterone and cAMP production was determined in cells incubated in the absence (open circles and squares respectively) or presence of 3.3 nmol LH/l (filled circles and squares respectively), with 3-isobutyl-1-methylxanthine (IBMX) (0.5mM) present throughout the preincubation and incubation periods. Results were normalized by considering the specific binding of ^{125}I-labelled hCG to cells from fractions F8 (49 ± 10.1 fmoles/ 10^6 Leydig cells) (a), and the response of the cells from fraction F8 with respect to production of testosterone (610 ± 177 pmol/ 10^6 Leydig cells/ 2h) and cAMP (280 ± 77.7 pmol/ 10^6 Leydig cells/ 2h) as 100%. Values are presented as Means ± SD of three different experiments each performed in triplicate. *P<0.05 compared with F4 and F5 (b), and F4, F5 and F6 (c) (one way analysis of variance). Basal production of testosterone (35 ± 17.6 pmol/ 10^6 Leydig cells/ 2h) and cAMP (7.02 ± 1.55 pmol/ 10^6 Leydig cells/ 2h) was similar in all fractions.


(a)
Binding of ^{125}I-labelled hCG (%)
120
100
80
60
40
20
0
F4
F5
F6
F7
F8
(b)
Testosterone production (%)
120
100
80
60
40
20
0
*
*
F4
F5
F6
F7
F8
(c)
cAMP production (%)
160
140
120
100
80
60
40
20
0
*
*
F4
F5
F6
F7
F8
Fraction number

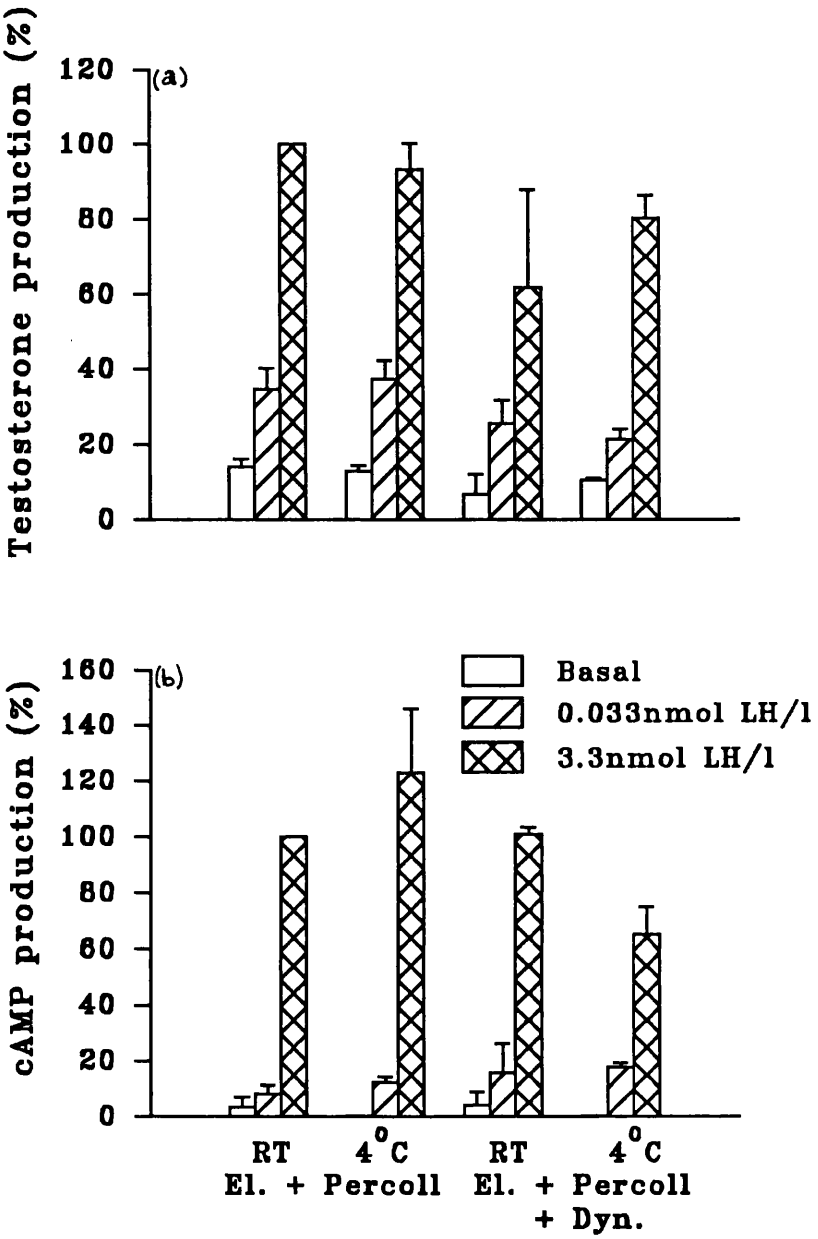


Figure 3.4. Basal and LH-stimulated testosterone (a) and cAMP (b) production by elutriated/ Percoll-purified Leydig cells (El. + Percoll) and Leydig cells further purified with Dynabeads (P-450) coated with a macrophage monoclonal antibody ED2 (EL. + Percoll + Dyn.). Cells were kept either at room temperature (RT) or at 4°C during purification with Dynabeads (M-450) for approximately 2h. The cells were incubated as described in Fig.2 in the absence or presence of 0.033 nmol or 3.3 nmol LH/l. Results were normalized by considering the response of cells to 3.3 nmol LH/l with respect to testosterone (402 ± 21.8 pmol/ 10^6 Leydig cells/ 2h) and cAMP (290 ± 9.2 pmol/ 10^6 Leydig cells/ 2h) production as 100%. Values are presented as means ± SD of 3 separate experiments each performed in triplicate.

Fraction	Viability (%)
Elutriated + Percoll (RT)	96.8 ± 1.36
Elutriated + Percoll (ice)	89.1 ± 5.19
Elutriated + Percoll (RT) + Dynabeads	97.0 ± 1.92
Elutriated + Percoll (ice) + Dynabeads	94.1 ± 1.10

Table 3.3. Viability of Leydig cells after elutriation/Percoll purification and after a further purification with Dynabeads M-450 coated with a macrophage monoclonal antibody. The percentage of viable cells was determined by diaphorase cytochemistry. Aliquots from the elutriated/ Percoll-purified fraction corresponding to fractions F4 to F8 kept at room temperature (RT) or on ice and from the same fraction further purified with Dynabeads (M-450) and kept in the same conditions (RT or ice) were analysed for the viability. The percentage of viable Leydig cells was determined by counting at least 5 fields of 500 cells each. Results are presented as means ± SD of 3 separate experiments, each performed in duplicate.

LH concentration (nmol/l)	Testosterone production	
	Fraction A	Fraction (A+B)
0	15.5 ± 1.00	14.6 ± 0.80
0.0033	36.2 ± 5.61	35.1 ± 0.50
3.3	100	106 ± 7.72

Table 3.4. Effect of macrophage enriched testicular interstitial cells on testosterone production by elutriated and Percoll-purified Leydig cells. Basal and LH-stimulated testosterone production by elutriated/Percoll-purified Leydig cells from the fraction corresponding to fractions F4 to F8 (band 2) containing 4.2 (± 1.58) % macrophages (Fraction A) and the same cell fraction to which cells from the macrophage-enriched fraction (Band 1 from the same fraction) containing 16.7 (± 3.57) % macrophages, 9.65 (±1.51) % Leydig cells and unidentified cells including germ cells (Fraction B) were added. The percentage of macrophages in the combined fraction (Fraction A+B) was 10.4%. The cells were incubated as described in Figure 3.3. Results were normalized by considering the response of the cells from Fraction A to 3.3 nmol LH/ml (412 ± 42.6 pmol/10^6 Leydig cells/2h) as 100%. Values from a representative experiment are presented as means ± SD of 4 different incubations each assayed in triplicate.

3.4. DISCUSSION

In the present study, a method has been investigated for preparing Leydig cells from rat testes which involves centrifugal elutriation in combination with Percoll density gradient centrifugation, with additional purification using magnetic beads coated with a macrophage monoclonal antibody. This has resulted in high yields of very pure viable Leydig cells virtually free from macrophages. Such highly purified Leydig cells will be useful for studying Leydig cell interactions with other testis cells, especially macrophages.

There is considerable confusion in the literature as to whether the heterogeneity of Leydig cells found by many workers is a result of the isolation procedures used (which may have damaged the cells) and/or reflects true functional differences. In the present study heterogeneity of Leydig cells was again observed; functional capacity was found to be related to sedimentation velocity, and cell volume. Leydig cells with the highest sedimentation velocities and cell volume were approximately 2 fold more responsive to LH in terms of testosterone and cAMP production compared with those of the lowest sedimentation velocities and cell volume. However, there was no difference in the capacity of the cells to bind ^{125}I-labelled hCG. All of these cells had the same average density (1.075 g/ml). In several other studies in which the Leydig cells were separated on Percoll (Cooke *et al.*, 1981; Aldred & Cooke, 1982) or Metrizamide (Payne *et al.*, 1980; O'Shaughnessy *et al.*, 1981; Chase & Payne, 1983) density gradients, the Leydig cells with a higher density (1.075 and 1.136 g/ml (Percoll and Metrizamide

respectively)) were found to be more responsive to LH than those with a lower density (1.045 and 1.101 g/ml (Percoll and metrizamide respectively)) and no differences in binding capacity to [^{125}I]-labelled hCG was found. We also found Leydig cells with a low density (1.045 g/ml, fraction F1-F3). However, because of their relative low Leydig cell purity they were not investigated further. Bhalla *et al.* (1987) and Browne *et al.* (1990) have demonstrated a Leydig cell functional heterogeneity associated with a difference in the binding capacity of the cells for ^{125}I-labelled hCG. Leydig cells with a lower density bound ^{125}I-labelled hCG with high affinity but were not steroidogenic in response to hCG, whereas, the Leydig cells with a higher density produced testosterone in response to hCG with little or no detectable ^{125}I-labelled hCG binding. The discrepancy between these findings and the results found in the present study and those reported by other workers could be due to the difference in the incubation temperature (37°C (Bhalla *et al.*, 1987; Browne *et al.*, 1990) and 4°C (the present study and others)) at which ^{125}I-labelled hCG binding was carried out, since the number of LH receptors per Leydig cell was found to vary widely depending on the temperature of incubation (Habberfield *et al.*, 1987). Also, the difference in the procedure of cell isolation might contribute to the differences in the parameters studied, because the capacity of the cells to produce cAMP and testosterone in response to hCG was very low in those studies compared to our findings and others. Leydig cell heterogeneity was not found by Dehejia *et al.* (1982), Aldred & Cooke (1983); Aquilano & Dufau (1984), Laws *et al.* (1985), Risbridger & de Kretser (1986) and Hedger & Eddy

(1987) who attributed the presence of the Leydig cell population with a lower density to cell damage and/or to Leydig cell precursors (mesenchymal cells). It was suggested that the Leydig cell heterogeneity is probably caused by the presence of protease(s) in partially purified preparations of collagenase used for dissociation of the interstitial cells (Aquilano & Dufau, 1984; Hedger & Eddy, 1987), which can cleave LH receptors (Ascoli & Segaloff, 1986). Several other lines of evidence suggest that increasing the severity of the dissociation conditions, by increasing the shaking frequency (Molenaar *et al.*, 1983) or prolonging the exposure of isolated cells to the dispersal conditions (Hedger & Eddy, 1987), causes an overall decline in the apparent buoyant density of Leydig cells and basal testosterone and hCG-stimulated testosterone and cAMP production.

The Leydig cell heterogeneity found in the present study is not due to cell damage nor to the presence of macrophages, since all the cells from the different fractions showed a similar viability and stained for 3ß-HSD. In addition, the presence of macrophages in the Leydig cell fractions does not appear to contribute to Leydig cell functional heterogeneity, since the macrophage content of the fractions rich in Leydig cells (F4 to F8), which were different with respect to sedimentation velocities and responsiveness to LH, was similar in all fractions (<3%). Also addition of macrophage-enriched interstitial cells had no effect on the responsiveness of the purified Leydig cells to LH. However, the latter observation needs further investigation, since other testicular cells including germ cells, which are known to regulate Leydig

cell steroidogenesis (See Skinner, 1991 for a review) were also present in the macrophage enriched-fraction.

Heterogeneity of testicular interstitial cell macrophages with respect to sedimentation velocities and densities found in the present study confirms previous findings in other tissues including lung, spleen, thymus, liver and bone marrow (Dijkstra *et al.*, 1985; Brannen & Chandler, 1988).

This study demonstrates the successful application of immunomagnetic particles, Dynabeads (M-450) coated with ED2, to obtain highly pure Leydig cell preparations virtually free from macrophages. The Leydig cells obtained by using this procedure of purification retained their viability and steroidogenic activity. The incubation temperature (room temperature or 4°C) had no effect on the degree of purity of Leydig cells nor on their responsiveness to LH. Although the removal of the macrophages did not have any significant effect on the responsiveness of the purified Leydig cells to LH, purification with the magnetic beads is necessary in studies in which the interaction of macrophages with Leydig cells is investigated and also when ligands which might affect the activity of macrophages are used. This method can also be used for the positive selection of macrophages (Lea *et al.*, 1986) and this will allow the characterization of testicular macrophages and the study of their interaction with Leydig cells and other testicular cells.

CHAPTER 4

EFFECTS OF A DOPAMINE AGONIST MESULERGINE (CU 32-085) ON RAT LEYDIG CELL FUNCTIONS

4.1. SUMMARY

The aim of the study was to investigate the changes in the biochemical properties of the Leydig cells which result from treatment *in vivo* with a dopamine agonist (CU 32-085).

Sprague Dawley rats (8 weeks old at the start of treatment) were treated with CU 32-085 (2mg/kg body weight/day) for 1, 5, or 12 weeks. The Leydig cells from control and treated rats were isolated by elutriation and density gradient centrifugation. The Leydig cells from rats treated for 5 weeks produced lower levels of testosterone in response to LH compared with the controls. The defect in steroidogenesis was associated with a decrease in the number of LH receptors and cAMP production. The decrease in LH receptors was detected as early as one week after treatment and was more pronounced after 5 and 12 weeks. The lesion in steroidogenesis was due to a defect in 17α-hydroxylase and 17-20 lyase, since pregnenolone production in response to 22R-hydroxycholesterol was not affected by the *in vivo* treatment with the DA while that of testosterone was decreased. This steroidogenic lesion which was previously shown to be caused by treatment with oestrogens was found in the present study to be associated with an increase in aromatase activity.

Preliminary evidence indicates that the DA may selectively inhibit steroidogenesis in the Leydig cells with lower sedimentation velocities.

When added *in vitro* to purified Leydig cells in primary culture, the DA inhibited testosterone production in response to LH at levels equal to or above 10^{-7}mol/l without having any inhibitory effect on cAMP production.

However, the effects of the compound on Leydig cells is more likely to result from the increase in circulating levels of LH, ACTH/glucocorticoids and the decrease in PRL. Since previous studies showed no binding of radiolabelled CU 32-085 to the testis and a preferential concentration of the metabolites of mesulergine in the striatum rather than in the plasma.

The decrease in the interstitial fluid volume as a result of treatment with the DA may be due to the local changes in testosterone and oestradiol levels. The latter were found not to be reflected by similar changes in the circulation.

4.2. INTRODUCTION

In rats the dopamine agonist (DA), mesulergine (CU 32-085), was found to cause a decrease in PRL release, an increase in circulating levels of LH and ACTH/glucocorticoids, and a decrease in growth hormone. The latter was only detected in rats treated with the DA for more than 4 weeks (Siegel & Prentice, 1987). The effects exerted by this DA were found to be mediated through its metabolites (e.g. 1,20-N, N-bidemethylated mesulergine) (Enz, 1981; Enz *et al.*, 1984; Marko, 1984). The inhibitory effect of dopamine and its agonists on PRL release has been suggested to be a result of a direct action on lactotrophs (Beck & Wuttke, 1977; Judd *et al.*, 1978). The role of the dopaminergic system in the regulation of LH secretion is controversial. Both stimulatory (Vijayan & McCann, 1978; Negrovilar, 1979) and inhibitory (Le Blanc *et al.*, 1976; Barnes *et al.*, 1986, 1987) effects have been demonstrated. In the rat, there is evidence that dopamine acts on α-adrenergic receptors to cause discharge of LHRH (Schneider & Mc Cann, 1969) and thus increase LH release. The changes in circulating pituitary hormones (LH, PRL and ACTH) caused by DA-treatment will affect the function of the target organs including testes and adrenals.

There is considerable evidence to support the concept of Leydig cell heterogeneity with respect to size and function (Payne *et al.*, 1980; Cooke *et al.*, 1981). Also paracrine control mechanisms exist in the testis which may involve interactions of the Leydig cells with other interstitial cell types and cells in the seminiferous tubules (see Sharpe, 1990; Skinner, 1991, for reviews).

Therefore, in the present investigation, the effects of treatment *in vivo* with the DA was assessed using elutriated/Percoll-purified Leydig cells and Leydig cells separated according to their different sizes. The functional parameters measured include LH receptors, cAMP, pregnenolone and testosterone production and aromatase activity. The direct effect of the DA on Leydig cell steroidogenesis was also investigated.

4.3. RESULTS

4.3.1. Food intake, body weight, testicular and seminal vesicle size, and interstitial fluid volume

At the dose used (2mg/kg body weight), the dopamine agonist (DA), mesulergine had no significant effect on food intake (Figure A2.1), body weight (Figure A2.2), testicular and seminal vesicle size (Figure A2.3), but significantly decreased testicular interstitial fluid volume (Figure A2.4).

4.3.2. Effect of *in vivo* treatment with CU 32-085 on LH receptors and steroidogenesis in rat Leydig cells

LH receptor studies were carried out on crude testicular interstitial cells and elutriated/Percoll-purified Leydig cells. Production of testosterone and cAMP and aromatase activity were determined using elutriated/Percoll-purified Leydig cells.

4.3.2.1. Specific binding of ^{125}I-labelled hCG to Leydig cells

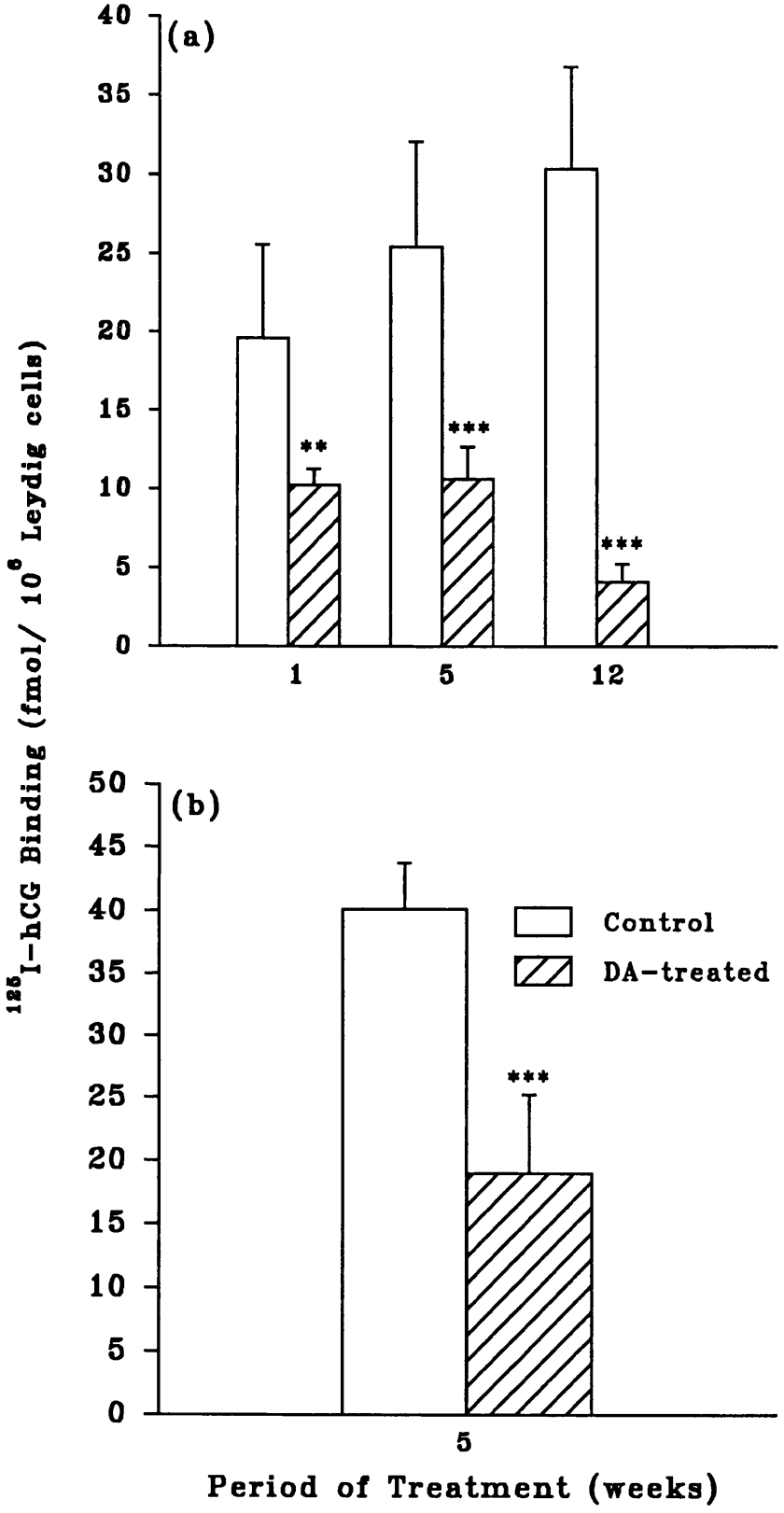
As shown in Figure 4.1, treatment with the DA resulted in a significant decrease in the specific binding of ^{125}I-labelled hCG to Leydig cells. This decrease was apparent in both crude (a) and purified Leydig cell preparations

(b). The decrease in the specific binding of ^{125}I-labelled hCG was detected as early as 1 week after DA treatment ($P<0.01$) and was more pronounced in the testes from the rats treated for 5 and 12 weeks ($P<0.001$). This effect is more likely due to a decrease in the number of LH/hCG receptors, since the affinity of the receptor to hCG was not altered by treatment with the DA as shown in Figure 4.2. Scatchard plot analysis indicated a single class of binding sites for the Leydig cells from both control and DA-treated rats with no apparent difference in the binding affinity. The dissociation constant (K_D) was 4.71×10^{-10}M for Leydig cell cells from controls and 4.65×10^{-10}M for those from the DA-treated group. Binding capacity was greater for control cells than the treated (32,519 and 22,883 receptors per Leydig cell in controls and DA-treated respectively).

Figure 4.1. Specific binding of ^{125}I-labelled hCG to Leydig cells in crude (a) and elutriated/Percoll-purified (b) testicular interstitial cells from testes of control and DA-treated rats.

^{125}I-labelled hCG binding in crude testicular interstitial cells (a) was determined by incubating one quarter of the interstitial cells from each testis from control rats and rats treated with the DA for 1, 5, or 12 weeks in LP4 tubes in 1ml DMEM containing 0.1% methyl cellulose. The binding of ^{125}I-labelled hCG to Leydig cells was assessed as described in Materials and Methods (Chapter 2) and expressed in fmol/10^6 Leydig cells. Results are presented as means ± SD of specific binding of ^{125}I-labelled hCG to Leydig cells of 5 to 6 individual animals each performed in triplicate.

^{125}I-labelled hCG binding in elutriated/Percoll-purified testicular interstitial cells (b) was determined by incubating purified cells obtained from control rats and rats treated with the DA for 5 weeks. The binding of ^{125}I-labelled hCG was determined as described in Materials and Methods (Chapter 2) and expressed in fmol/ 10^6 Leydig cells. Results are presented as means ± SD of 6 different experiments each performed in triplicate. **P<0.01 and ***P<0.001 compared with the controls of the same age.


(a)
40
35
30
25
20
15
10
5
0
**
***
***
1
5
12
(b)
50
45
40
35
30
25
20
15
10
5
0
***
Control
DA-treated
5
^{125}I-hCG Binding (fmol/ 10^6 Leydig cells)
Period of Treatment (weeks)

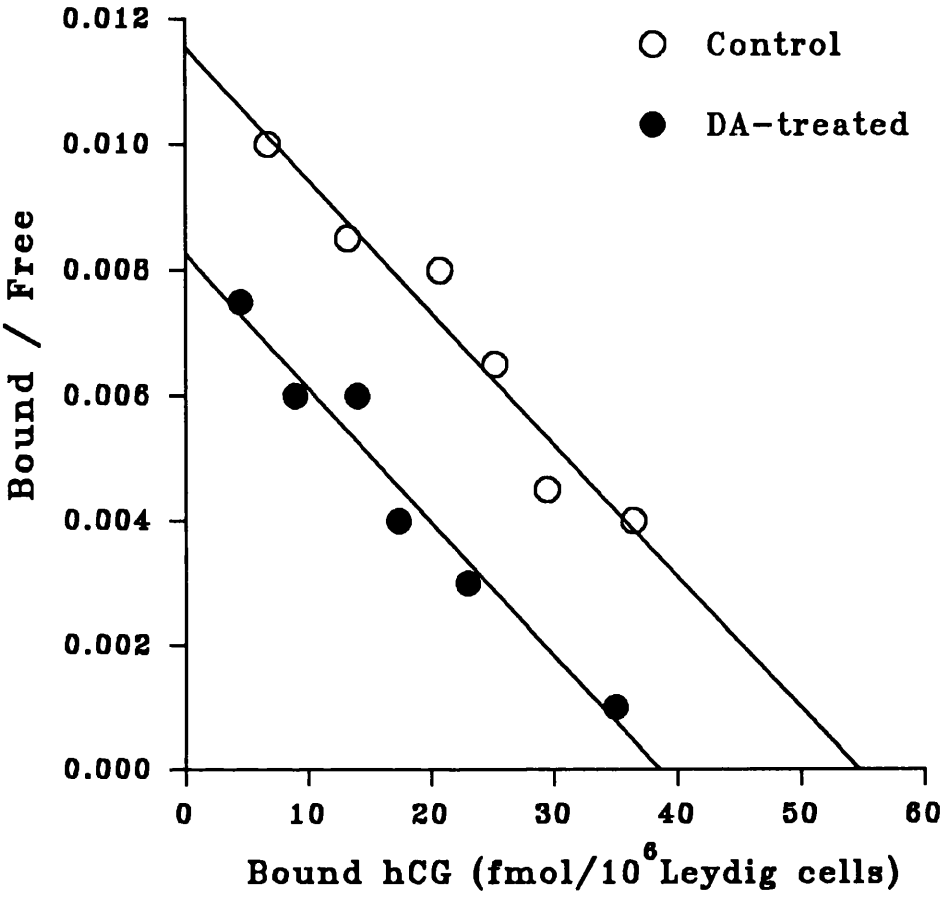


Figure 4.2. Scatchard analysis of the binding of ^{125}I-labelled hCG to elutriated/ Percoll purified Leydig cells from testes of controls and rats treated with the DA for 5 weeks.

The specific binding of ^{125}I-labelled hCG to Leydig cells was determined by incubating purified cells (10^6 cells) with increasing concentrations of ^{125}I-labelled hCG (0.55 to 8.33 nmol hCG/l) in the presence or absence of 27nmol unlabelled hCG/l as described in the Materials and Methods (Chapter 2). Levels of binding were determined and analysed for the best fit using the McPherson EBDA radioligand binding analysis programme. Results are presented as means ± SD of triplicate incubations.

4.3.2.2. Testosterone and cAMP production

As shown in Figure 4.3(a), treatment with the DA for 5 weeks caused a significant decrease ($P<0.01$) in basal testosterone production. The responsiveness of the Leydig cells to 0.0033 or 0.033 nmol LH/l, but not 3.3 nmol LH/l was significantly decreased as a result of *in vivo* treatment with the DA (Figure 4.3(a)). The decrease in the responsiveness of the Leydig cells to LH was more pronounced in the Leydig cells stimulated with the lowest concentration of LH (0.0033 nmol/l) ($P<0.001$) compared with that in response to 0.033 nmol LH/l ($P<0.01$).

The decrease in testosterone production in response to LH was found to be associated with a decrease in cAMP production (Figure 4.3(b)). The decrease in the responsiveness of the cells to LH with respect to cAMP production was found to be more pronounced in the cells stimulated with 0.033 and 0.33 nmol LH/l ($P<0.01$) compared with the cells stimulated with the highest concentration of LH (3.3 nmol/l) ($P<0.05$).

The responsiveness of the Leydig cells from testes of the DA-treated rats to 1mmol dcAMP was also significantly decreased ($P<0.01$) compared with the responsiveness of the Leydig cells from the controls. This suggests that the lesion in testosterone production cannot be only attributed to the decrease in LH receptors and cAMP production, another lesion in the steroidogenic pathway may occur as a result of treatment with this DA.

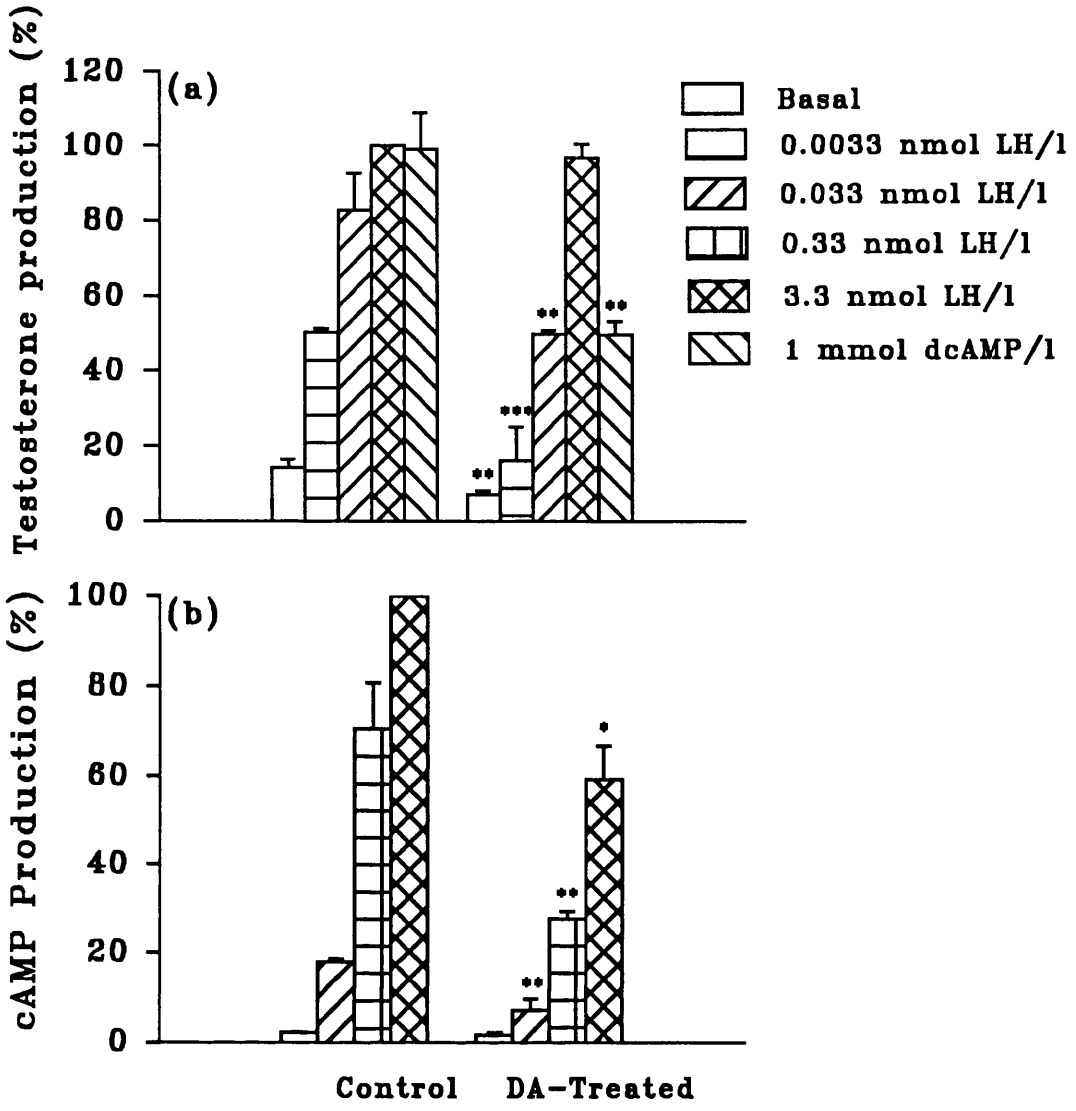


Figure 4.3. Testosterone (a) and cAMP (b) production in elutriated/Percoll-purified Leydig cells from testes of control rats and rats treated with the DA for 5 weeks.

Production of testosterone by Leydig cells was determined in Leydig cells (10^5 cells per well) incubated in 24 Costar well plates in the absence or presence of 0.0033, 0.033, or 3.3 nmol LH/l or 1 mmol dcAMP/l. Production of cAMP was assessed in Leydig cells incubated in the absence or presence of 0.033, 0.33, or 3.3 nmol LH/l. An inhibitor of cAMP metabolism, 0.05 mmol IBMX /l, was present throughout the preincubation and the incubation periods. Results were normalized by considering the response of the cells from the controls to 3.3 nmol LH/l with respect to testosterone production (a) (225 ± 10.6 pmol/10^6 Leydig cells/2h) and cAMP production (226 ± 22.6 (b), 172 ± 22.0 (c) pmol/10^6 Leydig cells/2h) as 100%. Values are presented as means ± SD of triplicate incubations each assayed in triplicate. *P<0.05, **P<0.01, and ***P<0.001 compared with corresponding controls.

4.3.2.3. The metabolism of 22R-hydroxycholesterol

In order to locate the lesion in the steroidogenic pathway, 22R-hydroxy-cholesterol (22R) was added to the Leydig cells from the control rats and rats treated with the DA for 5 weeks. It was found that the Leydig cells from testes of the treated animals had a much lower capacity ($P<0.01$ (0.5 µmol 22R/l) and $P<0.001$ (1 and 2 µmol 22R/l) to metabolise the 22R-hydroxy-cholesterol to testosterone (Figure 4.4(a)).

However when the metabolism of pregnenolone to testosterone was inhibited by the addition of 3ß-HSD and 17α-hydroxylase inhibitors, it was found that the formation of pregnenolone was not significantly different in the Leydig cells from testes of DA-treated rats compared with the Leydig cells from the controls (Figure 4.4(b)). This indicates that the lesion is in the enzyme(s) involved in the conversion of pregnenolone to testosterone and not the cholesterol side-chain cleavage enzyme.

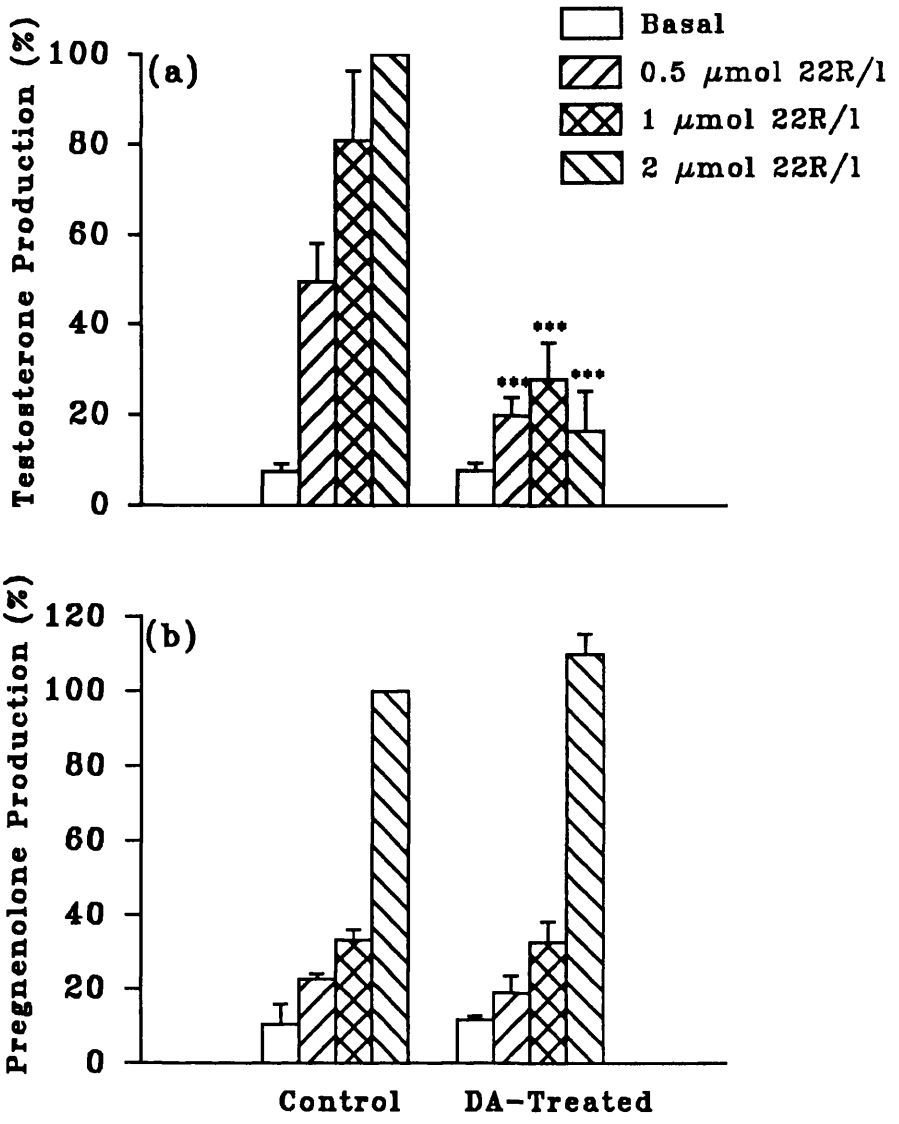


Figure 4.4. Testosterone (a) and pregnenolone (b) production in elutriated/ Percoll-purified Leydig cells from testes of control rats and rats treated with the DA for 5 weeks.

Production of testosterone and pregnenolone by Leydig cells was determined in Leydig cells (10^5 cells per well) incubated in 24 Costar well plates in the absence or presence of 0.5, 1, or 2 μmol 22R-hydroxycholesterol/l (22R). Inhibitors of pregnenolone metabolism were added 30min before addition of 22R at concentrations of 5 μmol and 20 μmol/l for cyanoketone and SU-10603-10603 respectively. Results were normalized by considering the response of the cells from the controls to 2 μmol 22R with respect to testosterone (742 ± 86.3 pmol/ 10^6 Leydig cells/2h) and pregnenolone (761 ± 22.2 pmol/ 10^6 Leydig cells/2h) as 100%. Results are presented as means ± SD of triplicate incubations each assayed in triplicate. ***P<0.001 compared with the corresponding controls.

4.3.2.4. Aromatase activity

Since, a lesion in testosterone production, similar to that caused by the DA was reported to occur in Leydig cells from rats treated with oestrogens, aromatase activity was measured in the Leydig cells from testes of the control rats and rats treated with the DA.

As shown in Figure 4.5, it was found that there was a significant increase ($P<0.001$) in aromatase activity after treatment with the DA. This indicates that the DA treatment causes an increase in oestrogen production by Leydig cells. The aromatase activity of Leydig cells from controls and DA-treated was inhibited *in vitro* by the addition of the aromatase inhibitor, 4-hydroxy-androstenedione (4OHA).

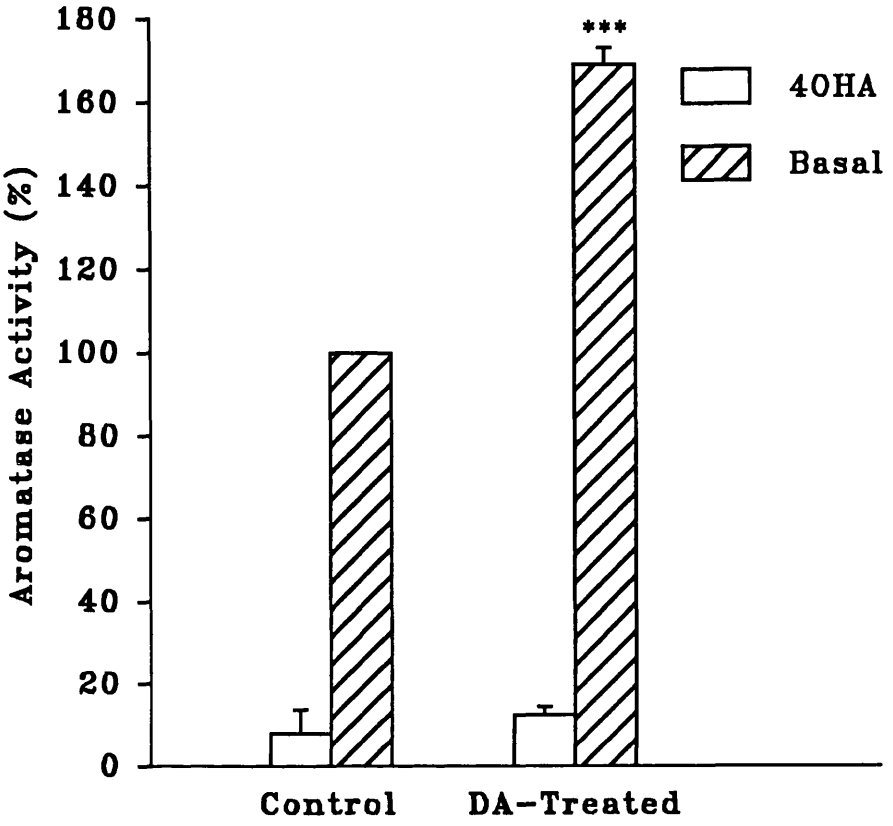


Figure 4.5. Aromatase activity in elutriated/Percoll purified testicular interstitial cells from testes of control rats and rats treated with the dopamine agonist (CU 32-085) for 5 weeks.

Aromatase activity was assessed by measuring ^3H_2O resulting from the aromatization of [1,2,6,7-^{3}H]-androstenedione. Purified Leydig cells (4 x 10^5 cells per well), from control rats and rats treated with the DA, were incubated in 1ml DMEM at 34°C in an air incubator in the absence or presence of 100µmol 4-hydroxyandrostenedione (4OHA)/l for 30min before the substrate (1,2,6,7-^{3}H]-androstenedione (0.2 µCi) was added. After addition of the substrate, the cells were incubated for 2h and the reaction was stopped by adding 100µl ice-cold 33.3% TCA. The non-specific activity was determined by stopping the reaction before adding the substrate. Aromatase activity was normalized by considering basal aromatase activity in cells from the controls (11979 ± 822 dpm/10^6 Leydig cells/2h) as 100%. Results are presented as means ± SD of 3 different experiments each performed in triplicate. ***P<0.001 compared with the corresponding control.

4.3.3. Effect of *in vivo* treatment with CU 32-085 on LH receptors and steroidogenesis in elutriated/Percoll-purified Leydig cells separated according to their different sedimentation velocities

There is a considerable evidence suggesting that the rat Leydig cells are heterogeneous with respect to their size, density and function. Therefore, the possible differential effect(s) of the DA on the different populations of Leydig cells was investigated. Leydig cells from testes of control rats and rats treated with the DA for 5 weeks were separated according to their sedimentation velocities by centrifugal elutriation in combination with Percoll gradient centrifugation. As shown in Figure 4.6 (a), treatment with the DA caused a decrease in the specific binding of ^{125}I-labelled hCG to Leydig cells of different sedimentation velocities in fraction F4 to F8. This effect was more pronounced in the Leydig cells from fraction F4 ($P<0.001$) and F7 ($P<0.01$) compared with those from fraction F5, F6 & F8 ($P<0.05$).

Treatment with the DA had a differential effect on basal and LH-stimulated testosterone production (Figure 4.6(b)). It significantly decreased the basal levels of testosterone produced by Leydig cells from fraction F4 and F5 ($P<0.001$) and increased basal levels of Leydig cells from fraction F6 to F8. The differential effect of the DA on the Leydig cells with different sedimentation velocities was also apparent in the cells stimulated with 3.3 nmol LH/l. It was found that only the Leydig cells with the lowest sedimentation velocities from fractions F4 and F5 had a lower capacity to synthesize testosterone when stimulated with LH compared with the

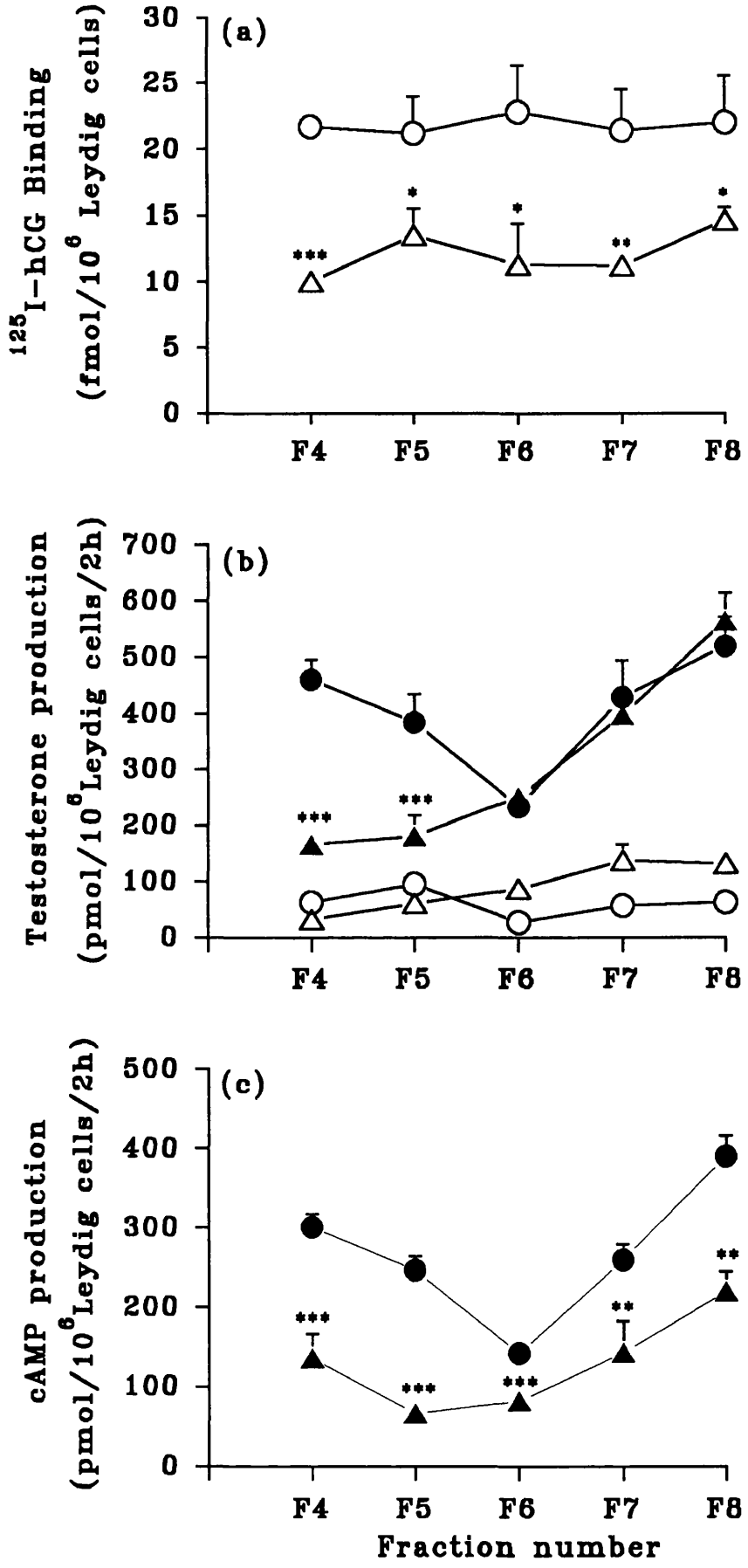
corresponding cell fractions from the controls ($P<0.001$). There is no significant difference between the Leydig cells from fraction F6 to F8 with respect to the capacity to produce testosterone when stimulated with 3.3 nmol LH/l in both control and DA-treated rats. However, the responsiveness of the Leydig cells from fraction F6 to F8 to LH with respect to testosterone production was decreased as a result of treatment with the DA, since higher basal levels of testosterone were produced by the Leydig cells from the same fractions from the treated animals compared with those from the controls.

The decrease in the responsiveness of the Leydig cells from fraction F4 to F8 to 3.3 nmol LH/l with respect to testosterone production was found to be associated with a parallel decrease in cAMP production by those cells (Figure 4.6(c)). This effect was more pronounced in the Leydig cells from fraction F4 to F6 ($P<0.001$) compared with those from fractions F7 and F8 ($P<0.01$).

The effect of the DA on basal levels of cAMP in the different cell fractions is not shown because it was undetectable as a result of the large volume of the condition medium and the limit of detectability of the assay. The functional heterogeneity of the Leydig cells within the control and DA-treated group, and the differential effect of the *in vivo* treatment with the DA on the responsiveness of the Leydig cells to LH with respect to testosterone and cAMP production cannot be attributed to a difference in Leydig cell content or cell viability, since the two parameters are not significantly different in the Leydig cells from fraction F4 to F8 from both groups (Figure 4.7 and Table 4.1) and they were considered for the final results.

Figure 4.6. Specific binding of ^{125}I-labelled hCG to Leydig cells (a) and production of testosterone (b) and cAMP (c) in elutriated/Percoll-purified interstitial cells, from testes of control rats and rats treated with the DA for 5 weeks, separated according to their different sedimentation velocities.

Elutriated/Percoll-purified Leydig cells from fractions F4 to F8, from control rats (circles) and rats treated with the DA for 4 weeks (triangles), were incubated in Costar 24 well plates at a cell density of 1.5 x 10^5 cells per well (^{125}I-labelled hCG) and 10^5 cells per well (testosterone and cAMP production). Binding of ^{125}I-labelled hCG to Leydig cells was determined as described in Figure 4.1. and expressed in fmol/10^6 Leydig cells. Production of testosterone and cAMP was determined in cells incubated in the absence (open circles and triangles) or presence of 3.3 nmol LH/l (filled circles and triangles, in controls and DA-treated respectively), with 0.5 mmol IBMX/l present throughout the preincubation and incubation periods. Results are expressed as pmol/10^6 Leydig cells/2h. Values are presented as means ± SD of triplicate incubations. *P<0.05, **p<0.01, and ***P<0.001 compared with the corresponding controls. Basal testosterone production by the Leydig cells from testes of the DA-treated rats was significantly lower than that produced by the corresponding controls in fractions F4 and F5 (P<0.001). Higher basal levels were produced by the Leydig cells from the treated animals in fractions F6 to F8 (P<0.001) compared with the corresponding controls.


(a)
^{125}I-hCG Binding
(fmol/10^6 Leydig cells)
30
25
20
15
10
5
0
F4
F5
F6
F7
F8
(b)
Testosterone production
(pmol/10^{6}Leydig cells/2h)
700
600
500
400
300
200
100
0
(c)
cAMP production
(pmol/10^{6}Leydig cells/2h)
500
400
300
200
100
0
Fraction number

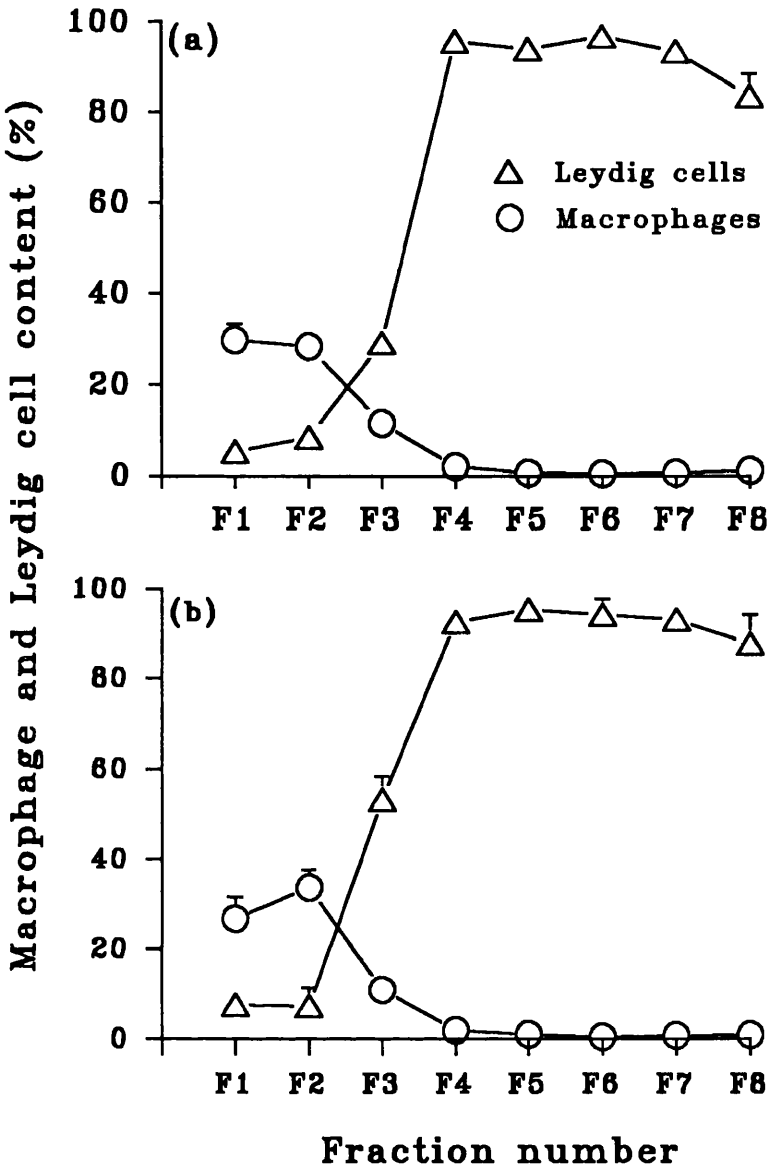


Figure 4.7. Macrophage and Leydig cell content in elutriated/Percoll-purified interstitial cell fractions from testes of control rats and rats treated with the DA for 5 weeks.

Aliquots from elutriated/Percoll-purified fractions (F1 to F8) from controls (a) and DA-treated rats (b) were assayed for Leydig cells and macrophages. The Leydig cells were identified by cytochemical staining for 3ß-HSD, and the macrophages were characterized by immunocytochemistry using a macrophage monoclonal antibody ED2. The percentage of Leydig cells and macrophages within each fraction were determined by counting at least 5 fields of 500 cells each. Results are presented as means ± SD of a representative experiment performed in triplicate.

Fraction Number	Viability	
	Control	DA-treated
F4	98.1 ± 1.22	95.9 ± 2.39
F5	96.5 ± 1.71	96.4 ± 0.88
F6	97.1 ± 0.78	96.9 ± 1.04
F7	95.4 ± 1.26	97.0 ± 0.58
F8	96.0 ± 0.60	97.7 ± 0.66

Table 4.1. Viability of elutriated/Percoll-purified Leydig cells, from testes of control rats and rats treated with the DA for 5 weeks, separated according to their different sedimentation velocities. The percentage of viable cells was determined by diaphorase cytochemistry. Aliquots from the elutriated/Percoll-purified fractions were analysed for the viability. The percentage of viable cells was determined by counting at least 5 fields of 500 cells each. Results are presented as means ± SD of a representative experiment performed in triplicate.

4.3.4. Effect of in vitro treatment with CU 32-085 on steroidogenesis in elutriated/Percoll-purified Leydig cells

Various concentrations of the dopamine agonist (DA) were added directly to the purified Leydig cells and the responsiveness of those cells to 3.3 nmol LH/l and 1 mmol dcAMP with respect to cAMP (LH only) and testosterone production was determined.

As shown in Figure 4.8(a), the DA had no significant effect on basal but inhibited testosterone production in response to 3.3 nmol of LH at levels equal to or above 10^{-7} mol DA/l, with the effect being more pronounced at 10^{-5} mol DA/l ($P<0.01$) compared with that at 10^{-7} and 10^{-6} mol DA/l ($P<0.05$).

However, the change in testosterone production in response to LH as a result of treatment *in vitro* with the DA was not associated with any significant change in cAMP production in response to the same concentration of LH (Figure 4.8(b)). Furthermore, although the DA did not alter the basal production of testosterone, basal cAMP production was increased at levels of 10^{-6} and 10^{-7} mol DA/l ($P<0.05$).

Since testosterone production in response to dcAMP was also decreased as a result of treatment *in vitro* with 10^{-5} mol DA/l (Figure 4.8 (a)), the inhibitory effect of the DA at higher concentrations on Leydig cell testosterone production is more likely to be exerted at a site beyond cAMP production.

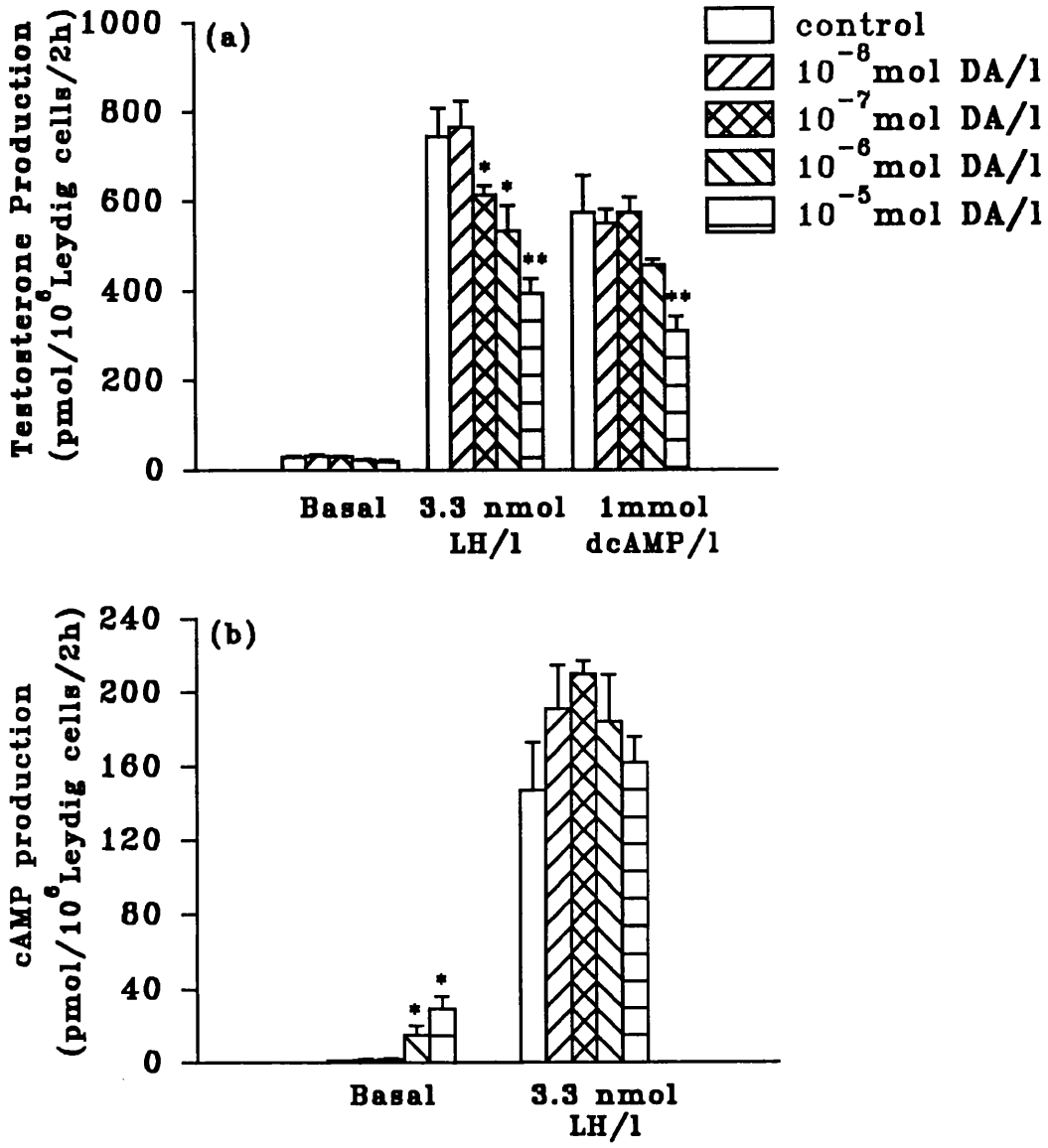


Figure. 4.8. *In vitro* effects of the DA on testosterone (a) and cAMP (b) production in elutriated/Percoll-purified Leydig cells from rat testes.
Elutriated/Percoll-purified Leydig cells were plated into Costar 24 well plates (10^5 cells per well). The cells were preincubated for 2h, then incubated for 2h in the absence or presence of DA added at different concentrations (10^{-8} to 10^{-5} mol DA/l) before 3.3 nmol LH/l or 1 mmol dcAMP/l were added. Testosterone and cAMP production are expressed in pmol/10^6 Leydig cells/2h. Results are presented as means ± SD of triplicate incubations. * $P<0.05$ and **$P<0.01$ compared with the corresponding control (basal, LH-, or dcAMP-stimulated).

4.4. DISCUSSION

Previous studies with the dopamine agonist (DA) (CU 32-085) (Siegel & Prentice, 1987) have demonstrated that this compound, when given acutely for 2 or 4h, causes a decrease in the secretion of LH, testosterone, PRL and growth hormone and an increase in the release of ACTH and glucocorticoids. The subacute study for 2 weeks, however, showed a decrease in PRL release without any change in the other hormones. Chronic studies for 4, 10, 13, 14, 17, 39, 53 and 66 weeks showed a significant increase in LH levels to 130 to 250% of control values together with a decrease in PRL and an increase in ACTH levels. From week 79 onwards, the increase in LH levels by the DA was no longer significant whereas PRL is always decreased. No changes in plasma concentrations of testosterone or oestrogens were detected at any of the times studied. However, the testosterone secretory response to a single injection of exogenous LH *in vivo* was found to be attenuated in the rats treated with the DA for 54 weeks. When similar studies were carried out on mice, no changes in circulating hormone levels were detected, suggesting a species difference in the action of this compound.

Using Leydig cells isolated from mature rats treated with the DA *in vivo*, we have extended these studies by investigating their biochemical properties *in vitro*. The decrease in ^{125}I-labelled hCG binding demonstrated in previous studies using testicular interstitial cell membranes (Siegel & Prentice, 1987), was confirmed in the present study using intact crude interstitial cells, elutriated/Percoll-purified Leydig cells, or Leydig cells fractionated according

to their sedimentation velocities. The decrease in the binding of ^{125}I-labelled hCG to Leydig cells from the treated animals was found to be due to a decrease in the number of receptors for LH/hCG rather than their affinity for LH/hCG. This was detectable as early as 1 week after treatment with the DA and was more pronounced at 5 and 12 weeks of treatment. This decrease in LH receptors occurred in all the Leydig cells separated according to their different sedimentation velocities. The decrease in LH receptors 1 week after DA treatment, which was not associated with any significant change in circulating LH levels, can be attributed to the decrease in circulating PRL levels. This is supported by previous studies which demonstrated that the number of LH/hCG receptors is modulated by PRL (Zipf *et al.*, 1978; Chan *et al.*, 1981; Klemke & Bartker, 1981). The decrease in LH receptors after 5 and 12 weeks of treatment with the DA, which was more pronounced compared with that detected after 1 week of treatment, can be due to the decrease in PRL as well as the increase in LH which causes down-regulation of these receptors. This is in agreement with previous studies in which chronic treatment with hCG *in vivo* was found to result in a loss in LH receptors without affecting their affinity to LH/hCG (Sharpe, 1976; Freeman & Ascoli, 1981). In addition, the increase in ACTH and thus glucocorticoid levels caused by treatment with the DA, may contribute to the decrease in LH receptors, since, there is evidence that treatment with glucocorticoids *in vivo* causes a decrease in the number of LH receptors in rat testes (Bambino & Hsueh, 1981). Possible changes in testicular factors as a result of DA treatment may

also be involved in the process of down regulation of the LH receptors. For example, there is evidence that TGFß (Avallet *et al.*, 1987) and IL1ß (Calkins *et al.*, 1988a) which are formed in the testis (Bicsak *et al.*, 1986; Khan *et al.*, 1987) cause a decrease in LH receptors.

The decrease in LH receptors as a result of treatment with the DA was accompanied by a decrease in the responses to LH in terms of cAMP and testosterone production. Treatment with the DA had a differential effect on the capacity of the Leydig cells to produce testosterone; the Leydig cells with lower sedimentation velocities produced less basal and LH-stimulated testosterone compared with their respective controls. The Leydig cells with higher sedimentation velocities, however, exhibited higher basal levels and a similar capacity to produce testosterone in response to LH compared with the corresponding controls. The decrease in the responsiveness of the Leydig cells from the treated rats cannot be attributed to the decrease in LH receptors and/or cAMP production, because the remaining receptors and the levels of cAMP produced by the Leydig cells from the treated animals are more than sufficient to maintain maximum steroidogenesis. In previous studies, it has been shown that only 1% of total LH receptors (Dufau *et al.*, 1975) and small levels of cAMP (Rommerts *et al.*, 1972; Dufau *et al.*, 1975) are needed to maintain Leydig cell steroidogenesis.

The production of pregnenolone in response to 22R-hydroxy-cholesterol was not affected by the *in vivo* treatment with the DA whereas that of testosterone was decreased. This indicates that the steroidogenic lesion was not in the

cholesterol side chain cleavage enzyme system, but probably in the 17α-hydroxylase/ C_{17-20} lyase. A similar lesion in steroidogenesis was found to result from *in vivo* treatment with LH/hCG (Nozu *et al.*, 1981; Oshaughnessy & Payne, 1982), and this was found to be associated with a decrease in the activity of 17α-hydroxylase and C_{17-20} lyase (Cigorraga *et al.*, 1978; Chasalow *et al.*, 1979; Dufau *et al.*, 1979). This was suggested to be caused by testicular 17ß-oestradiol (Purvis *et al.*, 1981) which was found to inhibit mRNA and enzyme synthesis (Nozu *et al.*, 1981; Ronco *et al.*, 1988). The involvement of oestradiol in this process was supported by many observations including increased production of oestradiol after LH/hCG treatment (Cigorraga *et al.*, 1980) and the presence of oestrogen receptors in rat testes (Brinkmann *et al.*, 1972). In addition, *in vivo* and *in vitro* treatment with oestrogens was found to cause a similar steroidogenic lesion (Brinkmann *et al.*, 1980; Melner & Abney, 1980; Moger, 1980). This steroidogenic defect was found to be preceded by an increase in Leydig cell aromatase activity (Valladares & Payne, 1979; Pomerantz, 1981; Tsai-Morris *et al.*, 1986, 1988). A similar block in steroidogenesis associated with high levels of oestrogens has been found in Leydig cell tumours (Samuels *et al.*, 1969; Orczyk *et al.*, 1987; Adams *et al.*, 1988). In the present study, the steroidogenic defect was also found to be associated with an increase in aromatase activity, thus indicating a potential for increased oestrogen production by the Leydig cells as a result of treatment with the DA. However, previous studies with the DA did not show any significant change in circulating oestradiol levels (Siegel & Prentice, 1987).

This could be due to the fact that 17ß-oestradiol levels were measured in peripheral venous blood rather than testicular venous blood or interstitial fluid. Since there is evidence that the changes in both interstitial fluid and testicular venous blood levels of testosterone do not always parallel those in peripheral venous blood (Maddocks & Setshell, 1989).

In addition to oestradiol, the high levels of corticosteroids caused by treatment with the DA (Siegel & Prentice, 1987) can also be responsible for this steroidogenic lesion, since there is evidence that *in vivo* treatment with corticosteroids causes a decrease in Leydig cell steroidogenesis (Bambino & Hsueh, 1981).

The functional changes caused by treatment with the DA can also involve other autocrine and/or paracrine factors. There is evidence that IL1-ß, which is one of the products of activated macrophages (Dinarello, 1984; Johnson, 1988) inhibits Leydig cell steroidogenesis and cAMP production (Calkins *et al.*, 1988). Other testicular factors including EGF (Hsueh *et al.*, 1981; Welsh & Hsueh, 1982) and TGFß (Avallet *et al.*, 1987; Lin *et al.*, 1987) may also contribute to the inhibitory effect on Leydig cell testosterone production exerted by the DA.

The possible direct effect of the DA on Leydig cell function was assessed *in vitro* using purified Leydig cells. It was found that the DA inhibited testosterone production in response to LH at levels equal to or above 10^{-7}mol/l without having any inhibitory effect on cAMP production in response to LH. Testosterone production in response to dcAMP was also decreased by the DA,

but only at a concentration of 10^{-5}mol/l. Basal cAMP production was increased in response to concentrations of DA equal to or above 10^{-6}mol/l but this was not associated with any change in basal testosterone production. The effect exerted by the DA *in vitro* on Leydig cells is more likely to be non-specific, since high concentrations of this compound are required and there is no evidence to support such effect. Therefore, it can be concluded that the changes in Leydig cell function caused by this dopamine agonist (CU 32-085) are more likely to result from the increase in circulating levels of LH, ACTH/glucocorticoids and the decrease in PRL, resulting from its effects on the central nervous system (Siegel & Prentice, 1987). This is supported by previous autoradiographic studies with [^{3}H]-CU 32-085, which did not show any detectable specific binding in the rat as well as mouse, dog, monkey and human testes (Siegel & Prentice, 1987).

The decrease in testicular interstitial fluid (IF) volume caused by the *in vivo* treatment with the DA can be due to the decrease and the increase in the local concentrations of testosterone and 17ß-oestradiol respectively. This is supported by a study by Widmark *et al.* (1987) which showed that chronic treatment of intact rats with 17ß-oestradiol decreases testicular IF volume. In addition testosterone was found to be required for the maintenance of IF volume in the adult rat testis and it was suggested that the seminiferous tubules may mediate this response through an androgen dependent mechanism (Maddocks & Sharpe, 1989).

CHAPTER 5

EFFECTS OF A DOPAMINE AGONIST MESULERGINE (CU 32-085) ON THE PROLIFERATION OF LEYDIG CELLS

5.1. SUMMARY

The dopamine agonist (DA) mesulergine (CU 32-085) was found to increase the incidence of Leydig cell tumours when given to rats for two and half years (Siegel & Prentice, 1987). The aim of the present study was to investigate the changes in Leydig cell growth and other related parameters which result from treatment with the DA in rats.

Sprague Dawley rats (8 weeks old) were treated with the DA (2mg/kg body weight/day) for 1, 2, 4, 5, 12, or 57 weeks. The DA treatment for 5 weeks caused an increase in the rate of protein synthesis by purified Leydig cells and an apparent increase in the number of Leydig cells with higher sedimentation velocities. This suggests a possible hypertrophic effect of the DA on Leydig cells. Treatment with the DA for 5 weeks did not have any significant effect on the number of Leydig cells and macrophages as determined using crude testicular interstitial cells or testicular sections. Also no significant change in the number of Leydig cells and macrophages was detected in rats treated for 1, 2, 4, and 12 weeks as measured in crude testicular interstitial cells. Treatment with the DA for 57 weeks caused a 42 and 31% increase in the number of Leydig cells and macrophages respectively.

Histological examination of testis sections from controls and DA-treated rats revealed the presence of nodules of Leydig cells surrounded in some parts by macrophages and fibroblast-like cells in the testes from the 57 but not the 5 week treated animals or the controls of both groups, although an increase in the number of Leydig cells occurred with ageing. Tubular atrophy was found

in some of the regions of the testis sections containing nodules of hyperplastic Leydig cells. Thick-walled arterioles were found in the intertubular spaces of the testis sections from the rats treated for 57 weeks.

Immunohistochemical examination of testis sections from control rats and rats treated with the DA for 57 weeks showed that inhibin-like immunoreactivity was detectable in both normal and tumour Leydig cells.

Based on these finding, those discussed in Chapter 4, and studies by other workers, it can be suggested that the effect of the DA on Leydig cell growth can be attributed to the changes in circulating LH, PRL, ACTH, and GH levels which resulted in the changes in the local testicular levels of testosterone, 17ß-oestradiol and other testicular factors. Therefore, the decrease in testosterone production and the increase in aromatase activity in Leydig cells isolated from the rats treated with the DA for 5 weeks, found in the present study, may be the cause for the development of Leydig cell tumours observed in the aged animals after long-term treatment with the DA.

5.2. INTRODUCTION

During the safety assessment of a dopamine agonist (DA) mesulergine (CU 32-085), it was found that the drug increased the incidence of Leydig cell tumours in a long-term ($2\frac{1}{2}$ years) carcinogenic study in rats (Table 5.1) but not in mice (Siegel & Prentice, 1987).

Rat Leydig cells are exceptionally sensitive to a wide variety of agents such as irradiation, carcinogenic hydrocarbons, copper, and zinc (see Mostofi & Bresler, 1976 for a review). In addition, Leydig cell proliferation is induced by hyperstimulation with gonadotrophic hormones (Courrier, 1966; Christensen & Peacock, 1980). A number of pharmaceutical compounds such as a calcium channel blocker (SDZ 200-110) (Roberts *et al.*, 1989), and the dopamine agonists (CF 25-397, an ergoline and AY 27-110, a nonergoline) (see Siegel & Prentice, 1987 for Refs) have also been shown to induce Leydig cell tumours in long-term studies in rats. Similar to CU 32-085 (Siegel & Prentice, 1987), SDZ 200-110 does not produce gonadal tumours in CD-1 mice of either sex or in female Sprague Dawley rats (Roberts *et al.*, 1989). Also the increase in the incidence of Leydig cell tumours in rats treated with this compound, like CU 32-085, was attributed to the increase in serum levels of gonadotrophins which was found to precede the appearance of the tumours.

Prolactin has also been implicated in the development of Leydig cell tumours; chronic hyperprolactinaemia, whether induced by ectopic pituitary transplants or chronic diethylstilbestrol treatment, was highly effective at reducing the incidence of Leydig cell tumours in Fischer 344 rats (Bartke *et al.*, 1985).

Group	Percentage of rats with Leydig cell tumours (%)
Controls (n=100)	5
DA-treated (n=50) (0.11mg/kg/day)	38
DA-treated (n=50) (0.42mg/kg/day)	80
DA-treated (n=50) (1.7mg/kg/day)	66

Table 5.1. Incidence of Leydig cell tumours in rats given the dopamine agonist (CU 32-085) for $2\frac{1}{2}$ years (Siegel & Prentice, 1987). n= number of animals.

This effect was suggested to be mediated via the control of gonadotrophin secretion (Carter & Whitehead, 1981; Cheung, 1983). Both acute (Smith & Bartke, 1987) and chronic (Hodson *et al.*, 1980) hyperprolactinaemia was found to suppress basal LH secretion with minimal effects on FSH. Cimetidine, an H2-receptor blocking agent was also found to increase the incidence of Leydig cell tumours (Fave *et al.*, 1977, Brimblecombe & Leslie, 1984) and this effect was attributed to its anti-androgenic action (Funder & Mercer, 1979, Baba *et al.*, 1981).

The mechanism of tumour development is still unclear. The possible involvement of testicular factors in this process cannot be excluded considering the interactions between the different types of testicular cells (see Sharpe, 1990 and Skinner, 1991 for reviews).

The aim of this study was to investigate the changes in Leydig cell growth and other related parameters which result from treatment with the DA in rats. The effect of the dopamine agonist (CU 32-085) on Leydig cell growth was determined by histological examination of testis sections. The number of Leydig cells and macrophages was determined using testis sections and also crude testicular interstitial cells. The possible hypertrophic effect of the DA on the Leydig cells was assessed by measuring the rate of protein synthesis and also by determining the distribition of the Leydig cells according to their sedimentation velocities. Immunohistochemical staining of testis sections with a polyclonal antibody against 58 kDa bovine inhibin was also determined.

5.3. RESULTS

5.3.1. Effect of treatment *in vivo* with the DA for 5 weeks on protein synthesis by elutriated/Percoll-purified Leydig cells

This was measured by the incorporation of ^{3}H-methionine into protein. As shown in Figure 5.1, treatment with the DA caused a significant increase in the rate of incorporation of ^{3}H-methionine into protein compared with the controls ($P<0.001$). The protein synthesis was completely inhibited by the addition of the translational inhibitor, cycloheximide, and partially inhibited by the addition of the transcriptional inhibitor, actinomycin-D. The increase in the rate of protein synthesis in the DA-treated animals is suggestive of a possible hypertrophic effect of this compound on Leydig cells.

5.3.2. Effect of treatment *in vivo* with the DA for 1, 2, 4, 5, or 12 weeks on the number of Leydig cells and macrophages in crude testicular interstitial cells

The effect of treatment with the DA on the number of Leydig cells and macrophages recovered after collagenase digestion of the testes was found to be variable as shown in Figure 5.2. The increase in the number of Leydig cells and macrophages detected in some experiments was found not to be statistically significant compared with the controls.

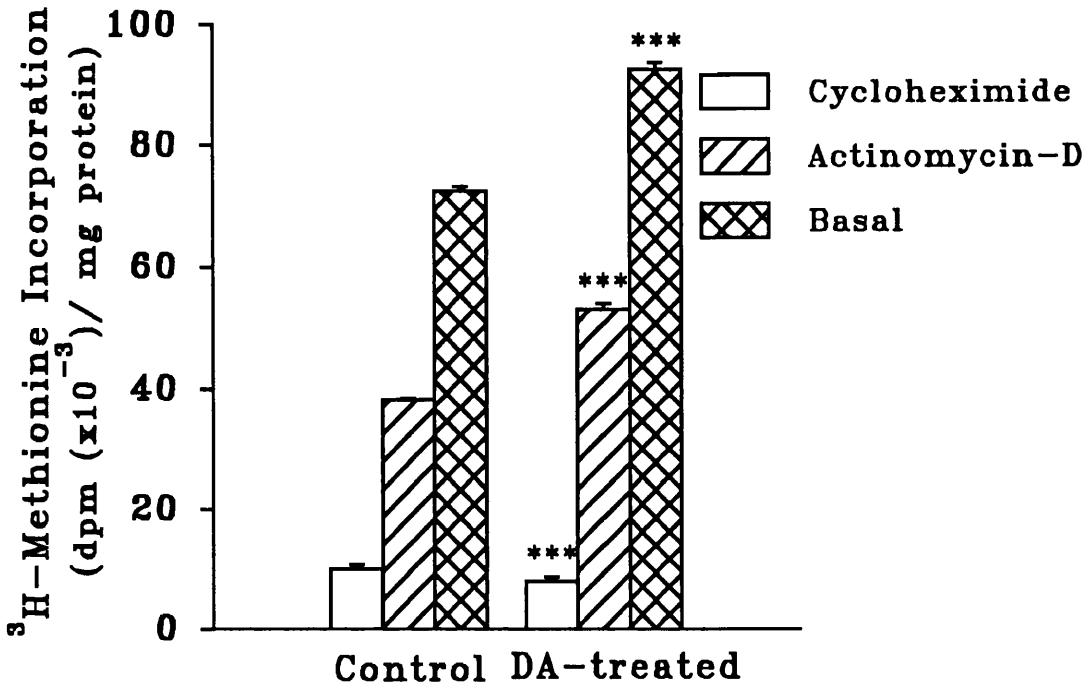


Figure 5.1. Protein synthesis in elutriated/Percoll-purified testicular interstitial cells in control and DA-treated rats.

Protein synthesis was determined by measuring the incorporation of ^{3}H-methionine into protein. Purified Leydig cells from control rats and rats treated with a DA for 5 weeks were plated into Costar 6 well plates (2 x 10^6 cells per well). After preincubation for 2h in DMEM/F12 containing 10mM unlabelled methionine, the cells were washed twice with 1ml methionine free media and recultured in 5ml methionine free media to which 5 µCi ^{3}H-methionine was added. Inhibitors of protein synthesis, actinomycin-D (5µg/ml) and cycloheximide (10µg/ml) were added 30min before ^{3}H-methionine was added. After a 4h incubation, the cells were washed twice with 5ml medium containing 10mM unlabelled methionine. The cells were then removed by scraping them with a rubber policeman and homogenized in 1ml PBS pH 7.4 containing 1% BSA and 10mM unlabelled methionine. Total protein (3 aliquots from each homogenate) was precipitated on GF/G filter with 10% TCA containing 10mM methionine. Protein synthesis is expressed in dpm ^{3}H-methionine incorporation/ mg protein/2h. The results are expressed as means ± SD of pooled data from two different experiments each performed in duplicate. ***P<0.001 compared with their respective controls.

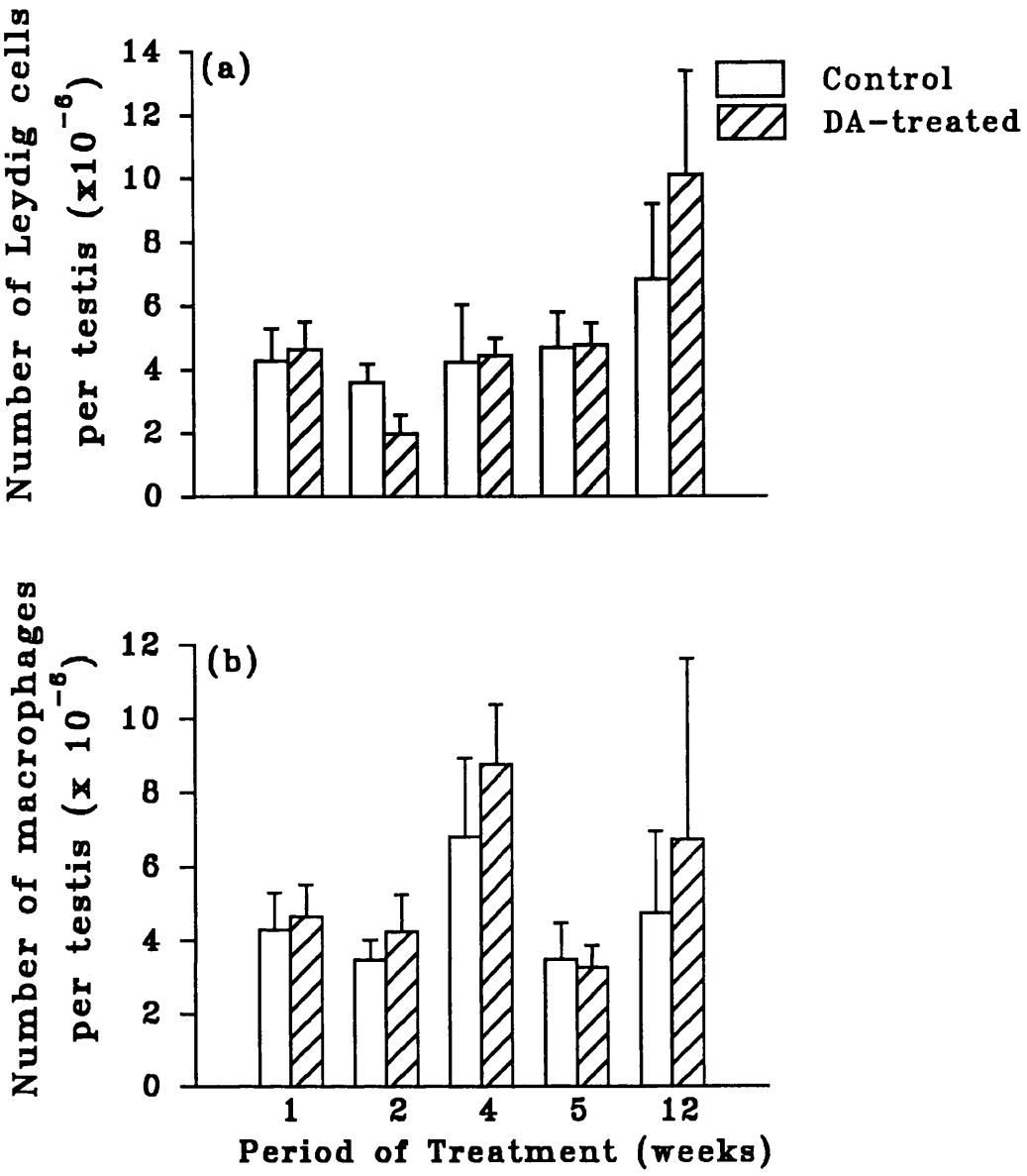


Figure 5.2. Number of Leydig cells and macrophages in crude testicular interstitial cells from controls and DA-treated rats.

Aliquots from crude testicular interstitial cells from paired testes of individual animals from the control rats and rats treated with the DA for 1, 2, 4, 5, and 12 weeks were assayed for Leydig cells (a) and macrophages (b). The Leydig cells were identified by cytochemical staining for 3ß-HSD, and the macrophages were characterized by immunocytochemistry using a macrophage monoclonal antibody ED2. The number of Leydig cells and macrophages was determined by counting at least 5 fields of 500 cells each. Results are presented as means ± SD, n= 5 to 6 animals per group.

5.3.3. Effect of treatment *in vivo* with the DA for 5 or 12 weeks on the distribution of Leydig cells according to their sedimentation velocities

The Leydig cells from the control rats and rats treated with the DA for 5 (Figure 5.3(a)) or 12 (Figure 5.3(b)) weeks showed different patterns of cell distribution according to their sedimentation velocities. Fractions F2, F3, and F5 from the rats treated for 5 weeks ($P<0.01$) and fractions F5, F6 and F7 from the animals treated for 12 weeks ($P<0.001$) contained more Leydig cells compared with those from the controls. Fraction F3 from the latter group had fewer Leydig cells compared with the same fraction from the control group. The increase in the number of Leydig cells with higher sedimentation velocities (Leydig cells from fractions F5 to F7) could be due to Leydig cell hypertrophy which resulted in an increase in cell size.

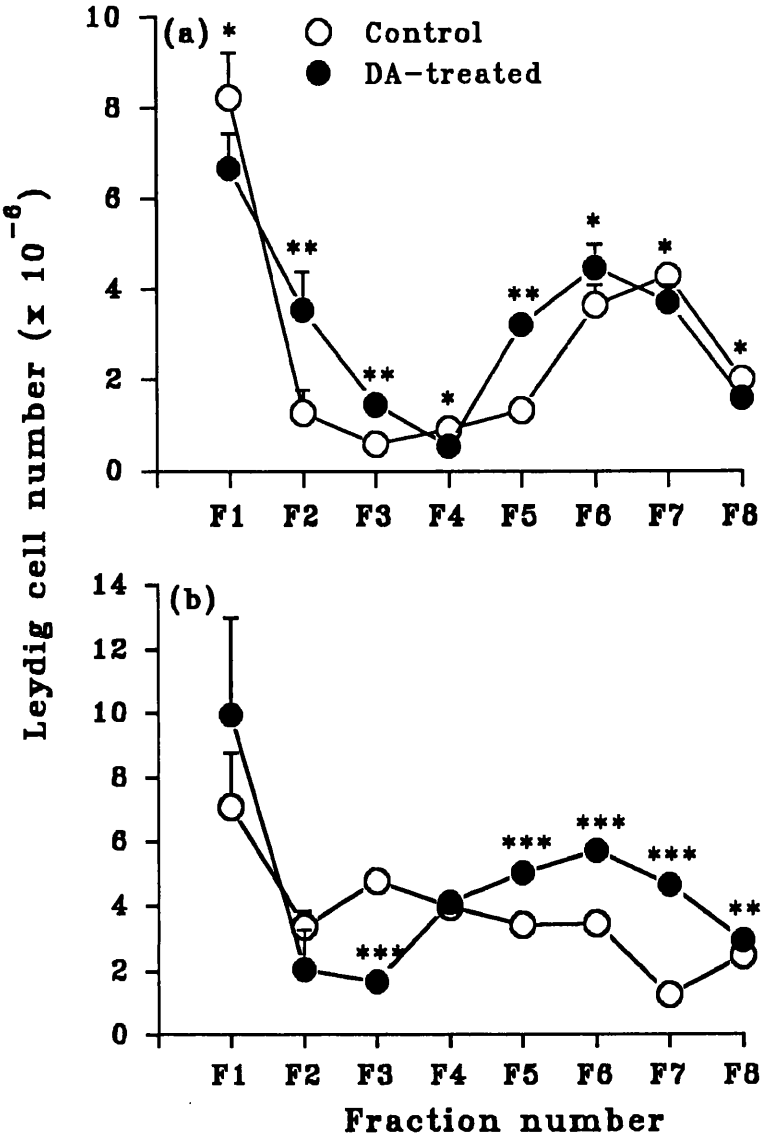


Figure 5.3. Leydig cell distribution according to their sedimentation velocities in control and DA-treated rats.

Crude interstitial cells from control rats and rats treated with the DA for 5 (a) or 12 (b) weeks were separated by centrifugal elutriation into 8 fractions according to their sedimentation velocities and then purified by density gradient centrifugation on Percoll. Aliquots from each fraction were analysed for 3ß-HSD. The number of Leydig cells was determined by counting at least 5 fields of 500 cells each. Results are presented as means ± SD of triplicate aliquots for each experiment. *P<0.05, **P<0.01 and ***P<0.001 compared with their respective controls.

5.3.4. Histological analysis of Leydig cell tumour development and determination of the number of Leydig cells and macrophages in testicular sections from control rats and rats treated *in vivo* with the DA for 5 or 57 weeks

Because of the variation in the results obtained in previous studies with respect to Leydig cell and macrophage content measured in isolated crude testicular interstitial cells, the number of macrophages and Leydig cells was determined using testicular sections to avoid any inaccuracy due to collagenase digestion an/or other factors.

As shown in Figure 5.4, treatment with the DA for 57 weeks but not 5 weeks resulted in a significant increase in the number of Leydig cells (a) and macrophages (b) ($P<0.001$). The aged rats (65 weeks old) exhibited a higher number of Leydig cells ($P<0.001$) and macrophages ($P<0.05$) compared with the youngest animals (13 weeks old). The relative numbers of Leydig cells, were 1,648 ± 128 and 2428 ± 277 in control and DA-treated rats of the oldest group, and 1,328 ± 114 and 1,266 ± 199 in control and DA-treated animals of the youngest group. The relative numbers of macrophages were 312 ± 27.3 and 409 ± 42.7 in the control and DA-treated rats of the oldest group, and 280 ± 27.5 and 281 ± 41.9 in control and DA-treated of the youngest group. No differences in the nuclear diameters of Leydig cells were found among the different groups (Table 5.2).

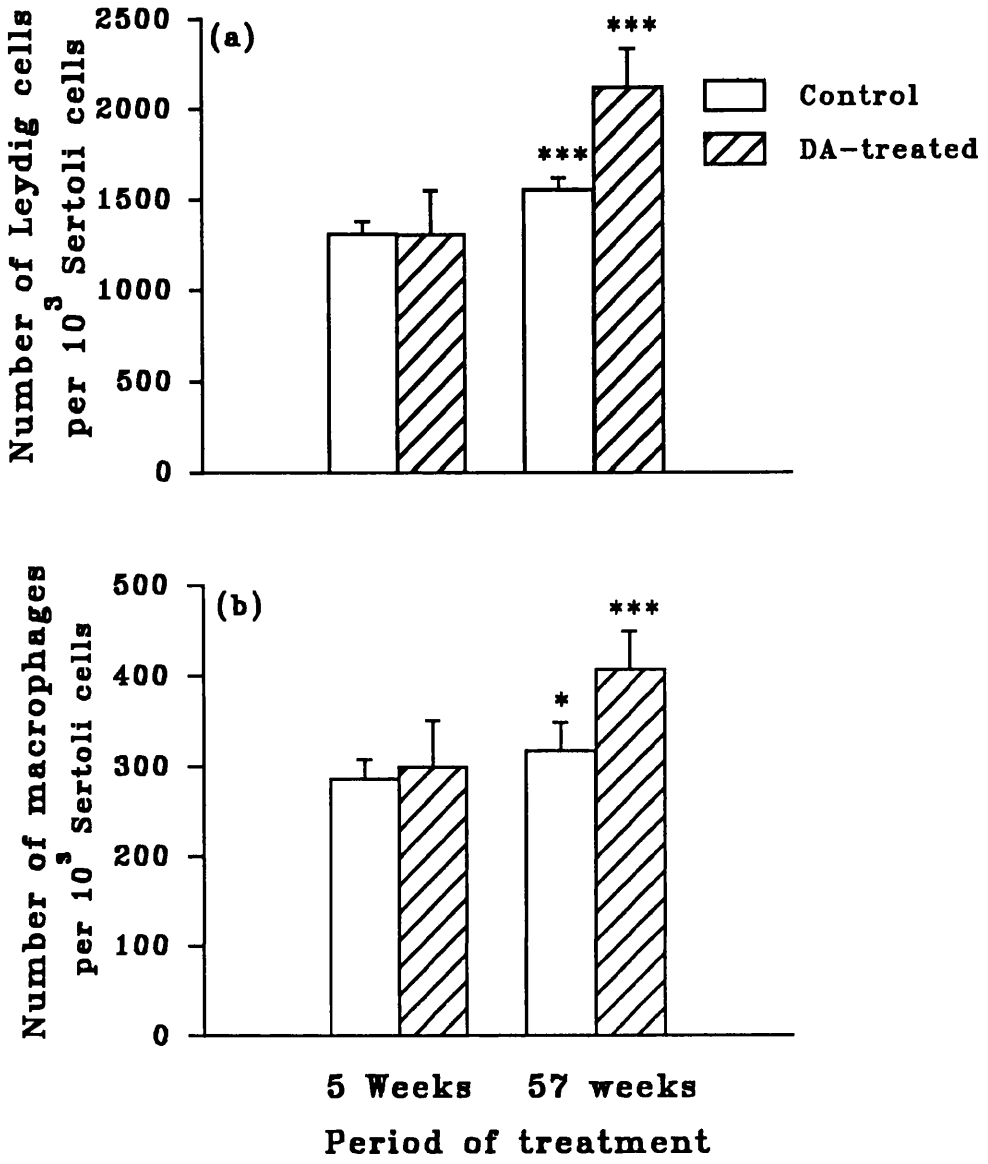


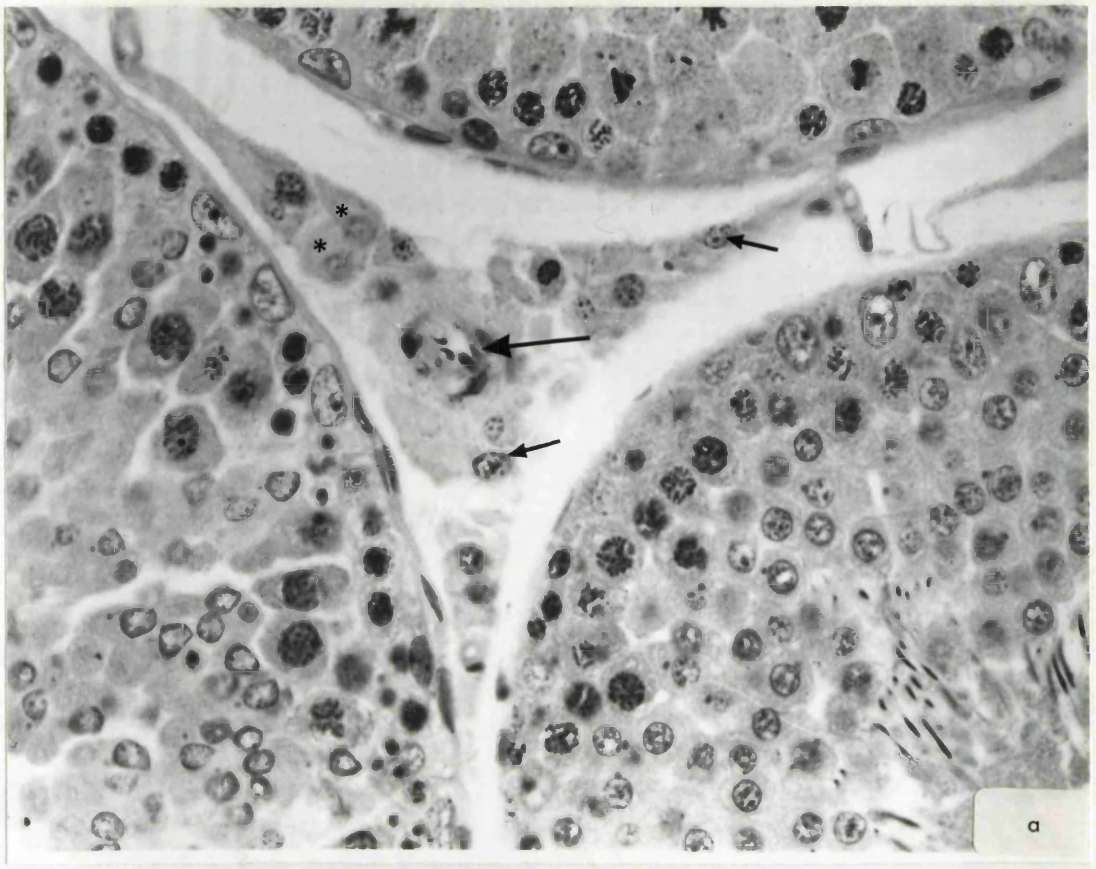
Figure 5.4. Number of Leydig cells and macrophages present in the interstitial tissue of testes from control and DA-treated rats. The number of Leydig cells (a) and macrophages (b) was determined using testicular sections from control rats and rats treated with the DA for 5 or 57 weeks as described in Materials and Methods (Chapter 2). The number of Leydig cells or macrophages is expressed per 1000 Sertoli cell nuclei. Results are presented as means ± SD of counts from 5 individual rats from each group. ***P<0.001 treated group for 57 weeks compared with the controls of the same age or the group treated for 5 weeks. *P<0.05 and ***P<0.001 oldest control group (64 weeks) compared with the youngest group (13 weeks) with respect to the number of macrophages and Leydig cells respectively.

Treatment	Mean nuclear diameter of Leydig cells (μm)	
	Normal areas	Nodular areas
Control (13 weeks old)	10.2 ± 1.04	_
DA-treated(5 weeks)	9.9 ± 0.63	_
Control (65 weeks old)	10.1 ± 0.87	_
DA-treated(57 weeks)	10.2 ± 0.79	9.3 ± 0.83

Table 5.2. Mean nuclear diameters of Leydig cells from control rats and rats treated with the DA for 5 or 57 weeks. The mean nuclear diameters of Leydig cells were determined as described by Teerds *et al.* (1989). Values are means ± SD of 60 nuclear diameters measured in sections from 3 animals (20 each) for each group. In the atypical areas 20 nuclear diameters of Leydig cells were measured and values represent mean ± SD.

No morphological changes have been detected in testis sections from the rats treated with the DA for 5 weeks compared with their respective controls (Figure 5.5a,b). In testes from the rats treated with the DA for 57 weeks, however, clusters (nodules) of tightly packed interstitial cells were found (Figure 5.5 c,d). The nuclear and cytoplasmic features of the cells in the nodules were characteristic of Leydig cells. The cells were medium sized with hexagonal cross sections and distinct cell boundaries, and possessed a regular round or oval nucleus, often with a nucleolus. Blood vessels were sometimes present within the Leydig cell nodules, but macrophages were always located around but not within the nodules. The nodules of Leydig cells were sometimes partly surrounded by cells with elongated nuclei and thin cytoplasmic extensions, possibly endothelial or fibroblast-like cells. In some of the sections, atrophic seminiferous tubules were found in the regions of the testis sections containing nodules of hyperplastic Leydig cells. Also thick-walled arterioles were present in the testis sections from the rats treated for 57 weeks.

Figure 5.5. Representative cross-sections of testes from 13-week-old control rats (a) and rats treated with the DA for 5 weeks (b). Blood vessels (large arrows), Leydig cells (small arrows), and macrophages (asterisks) are found in the interstitial tissue (magnification 571x).


a

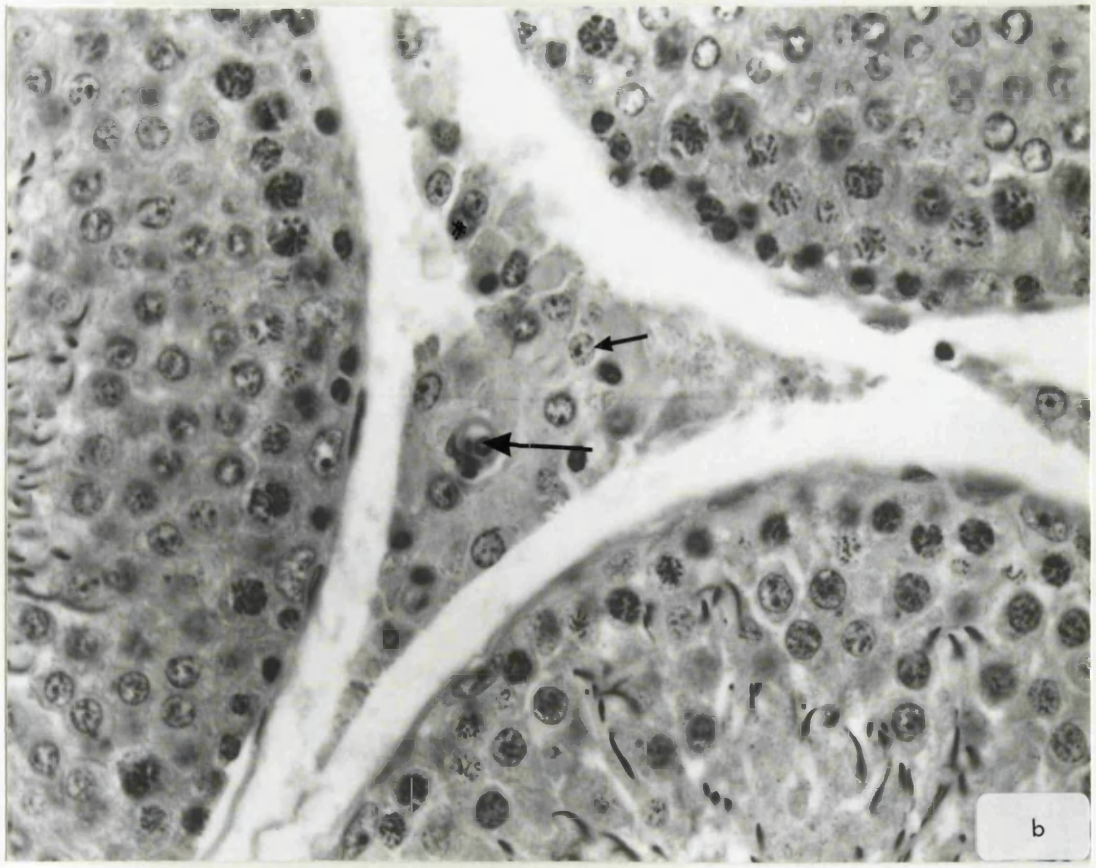
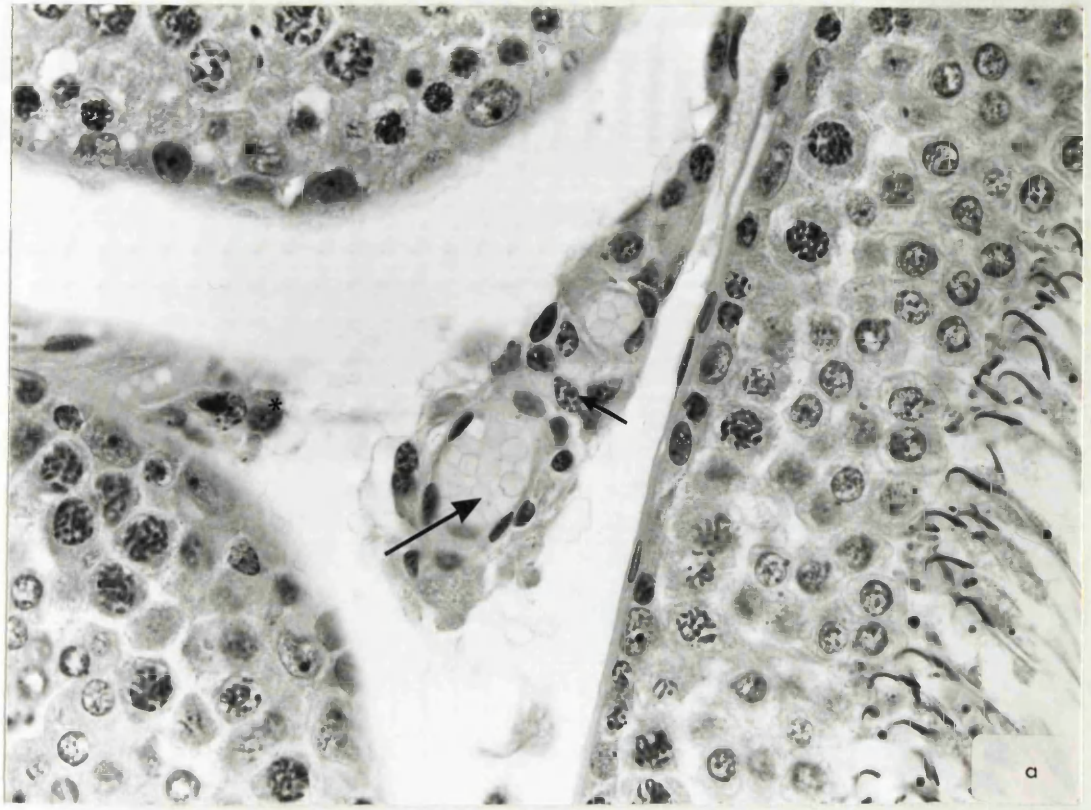
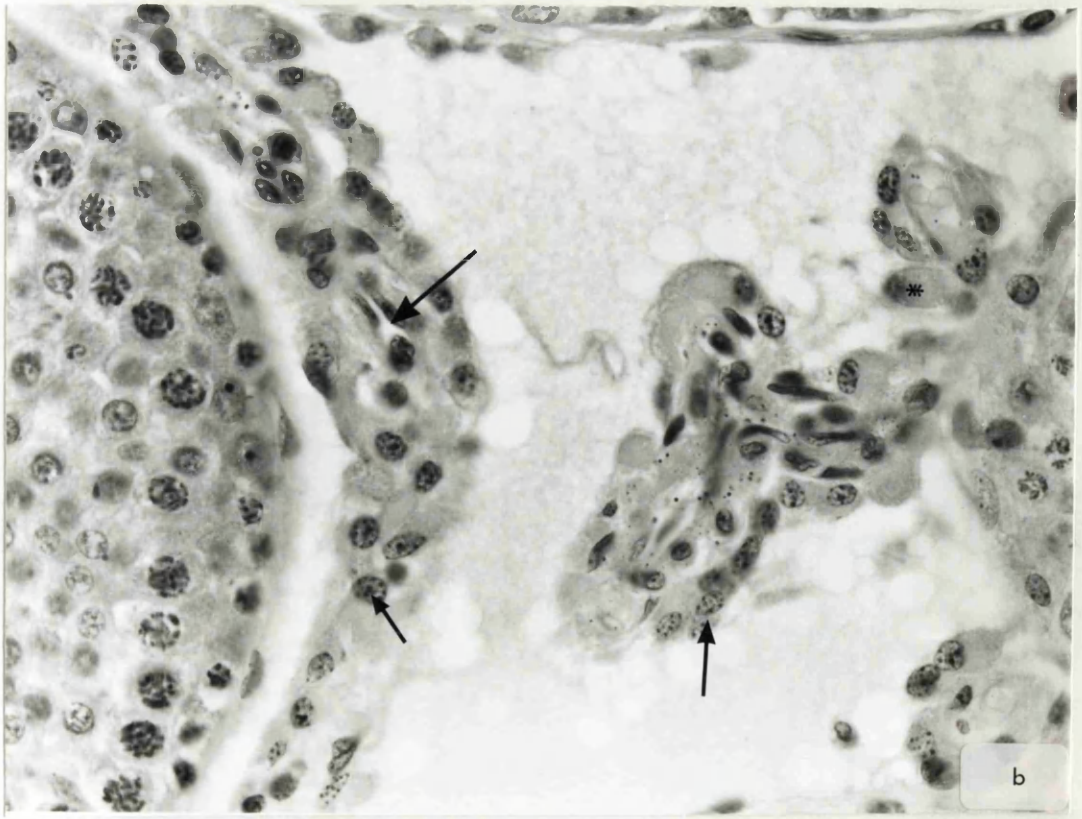
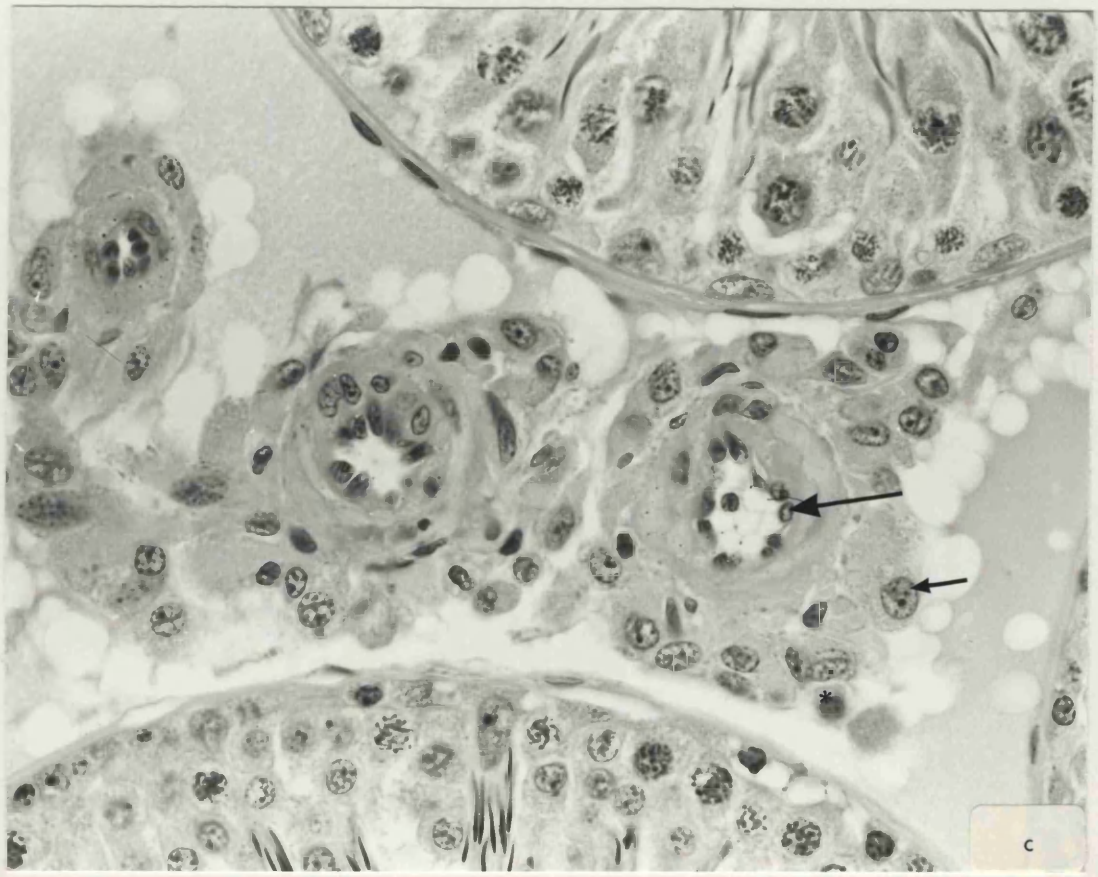
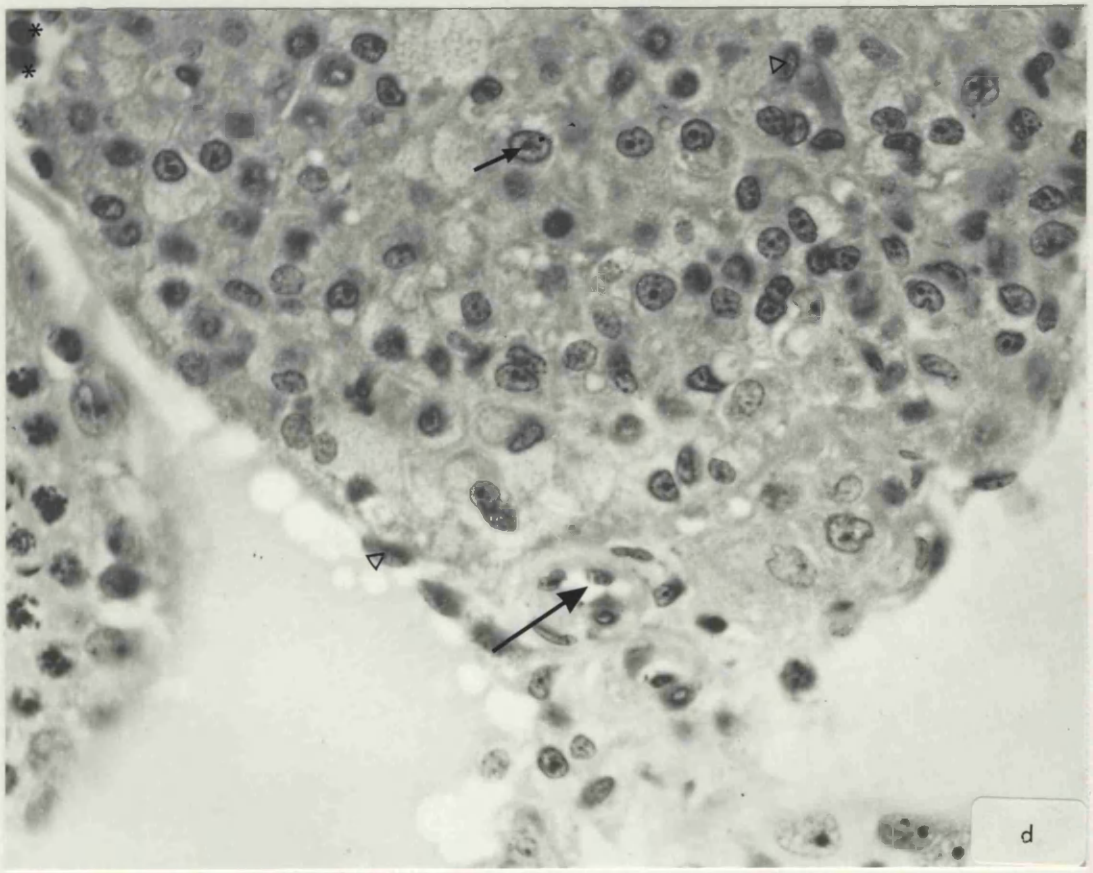

b

Figure 5.6. Representative cross-sections of testes from 65-week-old control rats (a,b) and rats treated with the DA for 57 weeks (c,d). Blood vessels (large arrows), Leydig cells (small arrows) and macrophages (asterisks) are found in the interstitial tissue. The section from one of the controls (b) shows a larger number of Leydig cells in the interstitium compared with the other controls of the same age and those aged 13 weeks (Figure 5.4a). In the sections from the DA-treated rats, clusters of tightly packed Leydig cells were frequently found in the interstitium and usually located around the blood vessels (c). Some of the nodules were very large reaching about the diameter of the seminiferous tubule; (d) represent part of a large nodule of Leydig cells. Fibroblast-like cells (empty triangles) are visible within and around the nodules of Leydig cells. Macrophages are always found around, but not between the Leydig cells within the nodules (magnification 571x (a,b,c) and 599x (d))


a


b

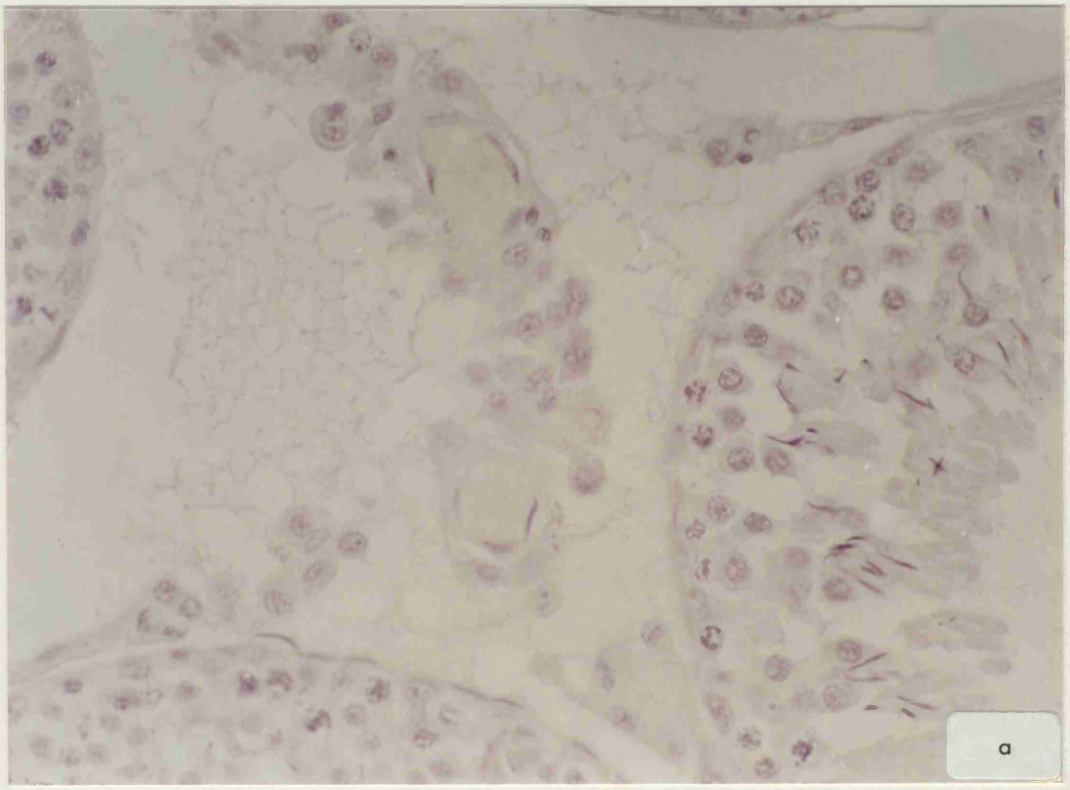

c

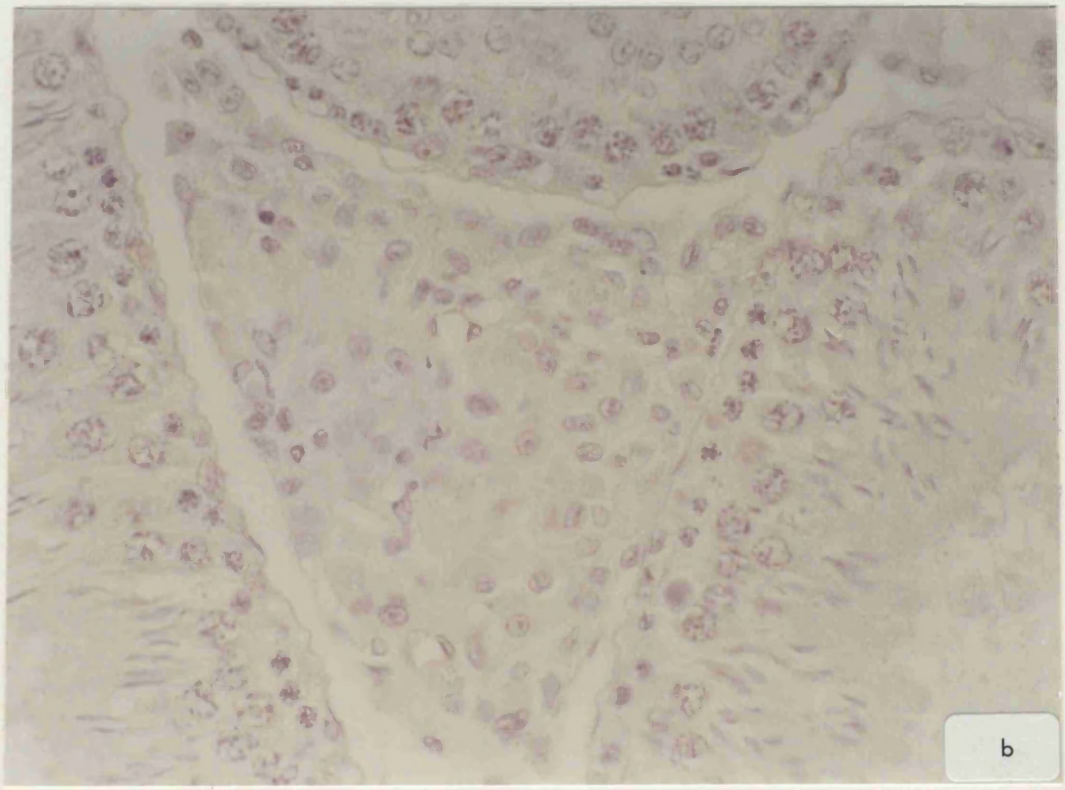

d

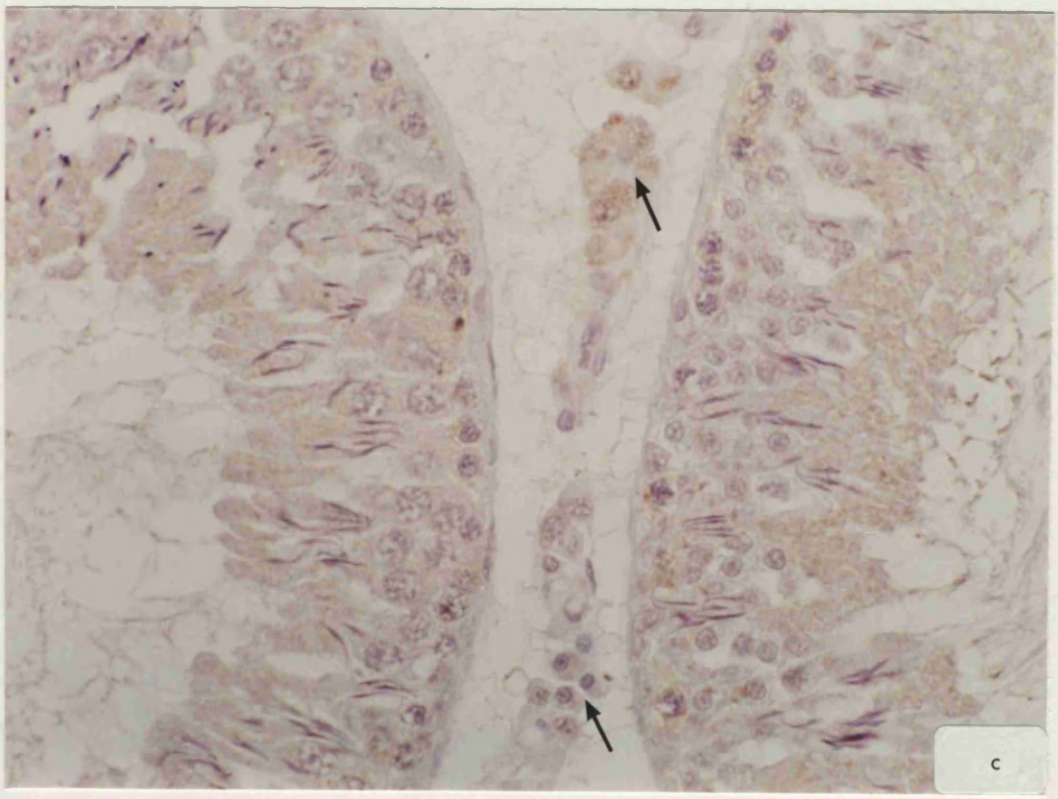
5.3.5. Inhibin-like immunoreactivity in testicular sections from control rats and rats treated *in vivo* with the DA for 57 weeks

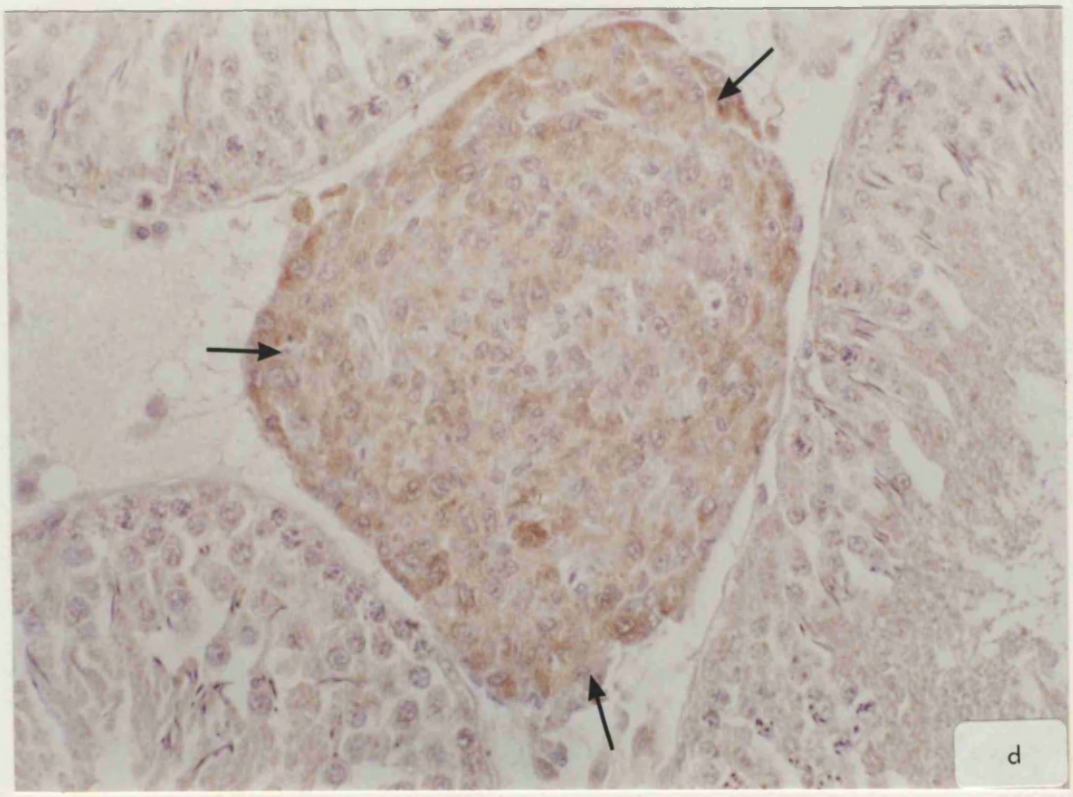
Immunohistochemical staining of paraffin embedded sections of 65 old control rats and rats treated with the DA for 57 weeks with a polyclonal antibody against 58 kDa bovine inhibin revealed that all Leydig cells (normal cells and cells located in nodules) showed inhibin-like immunoreactivity (Figure 5.7c,d). Faint positive staining was visible in the seminiferous tubules. The same distribution of inhibin-like immunoreactivity was found in testis sections from the untreated rats with no Leydig cell nodules. Whether the Leydig cells from the DA-treated animals produce more inhibin compared with those from the controls needs further investigation.

Figure 5.7. Inhibin-like immunoreactivity in representative cross sections of testes from controls and rats treated with the DA for 57 weeks. Non-specific staining with rabbit or bovine serum, or the second antibody (goat anti-rabbit immunoglobulin G) could not be detected (a,b in controls and DA-treated respectively). Inhibin staining is visible in the Leydig cells located in the interstitium of control rats (c) and in nodules (medium arrows) and in areas devoid of nodules in sections from the DA-treated rats (d). Faint staining was found in seminiferous tubules (magnification 500x (a,b,c) and 400x (d)).


a


b


c


d

5.4. DISCUSSION

In male rats, continuous treatment with the dopamine agonist CU 32-085, in the dose range capable of increasing the incidence of Leydig cell tumours, leads to chronically elevated circulating LH levels, an effect which first manifest itself following four weeks of drug administration and is sustained for most of the life time of the animals. In addition to the control of Leydig cell function, LH is implicated in the control of Leydig cell growth (Hodgson & Hudson, 1983). A possibility of a causal relationship between LH levels and development of Leydig cell tumours is strongly supported by results obtained by Brown *et al.* (1979) in parabiosed Fischer rats. In that study, chronic elevation of gonadotrophin produced by 20 months of parabiotic union with castrated males or ovariectomized females resulted in development of interstitial cell tumours. Furthermore, treatment with hCG (400 IU/rat) was found to result in Leydig cell hyperplasia three days after treatment in rats (Léon *et al.*, 1987). A similar effect was found in men who received 5,000 IU of hCG three times weekly for periods of forty seven to sixty-five days (Maddocks & Nelson, 1952). Therefore, it was suggested that the increase in the incidence of Leydig cell tumours caused by the CU 32-085 in the rat was caused by chronically elevated circulating LH levels (Siegel & Prentice, 1987). This is supported by the observation that structurally and pharmacologically different compounds (e.g. the calcium channel blocker, SDZ 200-110) (Roberts *et al.*, 1989), which induce Leydig cell tumours also leads to elevated serum LH levels in the rat. In addition, in CD-1 mice, where CU 32-085 did not

induce Leydig cell tumours, no changes in LH levels were detected (Siegel & Prentice, 1987). However, the time of appearance of Leydig cell tumours did not coincide with the increase in circulating LH levels; although the latter was detected as early as four weeks after treatment with the DA (Siegel & Prentice, 1987), the appearance of Leydig cell tumours only occurred in animals aged $2\frac{1}{2}$ years in which serum LH levels were no longer increased (Siegel & Prentice, 1987). This is in line with other studies in which elevated endogenous LH has been only implicated in the development of tumours in animals aged 18 to 24 months (Brown *et al.*, 1979; Bartke *et al.*, 1985). In contrast administration of exogenous gonadotrophic hormones can result in the formation of Leydig cell tumours within a short term after treatment (Léon *et al.*, 1987). This could be due to the high serum hormone levels achieved after treatment with LH/hCG in those studies.

Since the dopamine agonist, CU 32-085, exerted a suppressive effect on PRL secretion as demonstrated by Siegel & Prentice (1987), and other compounds with dopamine D2 agonistic properties, namely CF 25-397 and AY 27-110 also induce Leydig cell proliferation (see Siegel & Prentice, 1987 for Refs), it can be suggested that PRL may be involved either directly or indirectly in the development of Leydig cell tumours caused by CU 32-085. This is supported by the studies by Amador *et al.* (1985) and Bartke *et al.* (1985) which suggested that the development of Leydig cell tumours in aged Fischer rats may be caused by the decrease in circulating PRL levels.

Atrophy of the seminiferous tubules occurred in testis from the animals

treated with the DA for 57 weeks which contained nodules of Leydig cells. Thick-walled arteriols were also found in the testis sections from those animals. This is in line with studies in Fischer rats (Brown *et al.*, 1979; Turek & Desjardins, 1979; Bartke *et al.*, 1985), U-rats (Teerds *et al.*, 1991), and in human (Mineur *et al.*, 1987), in which involution of the seminiferous epithelium was found in all testes exhibiting extensive interstitial cell proliferation. Since the tubular atrophy was found to be confined to regions of the testis containing nodules of hyperplastic Leydig cells, it can be suggested that this was due to the local reduction in the availability of testosterone to the germinal epithelium and/or to the disruption of the local paracrine interactions between the germ cells, the Sertoli cells, and the Leydig cells (Bartke *et al.*, 1985).

A similar tubular damage was found to occur in rats treated with hCG and this effect was attributed to oestradiol, since this treatment was found to result in an increase in the circulating levels of this steroid (Léon *et al.*, 1987). Also intratesticular administration of oestradiol benzoate was able to produce the tubular damage caused by hCG treatment which leads to Leydig cell hyperplasia (Léon *et al.*, 1987). In the present study, treatment with the DA for 5 weeks was found to increase aromatase activity and thus 17ß-oestradiol production. This was not associated with any change in the number of Leydig cells, but hypertrophy of those cells cannot be excluded as demonstrated by the increase in the rate of protein synthesis and the number of the Leydig cells with higher sedimentation velocities (shift in Leydig cell

distribution). However, this latter observation needs an ultrastructural investigation.

Although no increase in the number of Leydig cells was detected after treatment with the DA for 5 weeks, the increase in the susceptibility of the Leydig cells to tumour induction caused by the DA when given at an earlier age to mature rats cannot be excluded. Because the induction of Leydig cell tumours was found not to occur in the aged rats in which treatment with the DA started at a later age (Siegel & Prentice, 1987).

The involvement of oestradiol in the development of Leydig cell tumours in the rat was supported by many observations which demonstrated the association of the appearance of Leydig cell tumours with high circulating levels of oestradiol (Turek & Desjardins, 1979; Mordes *et al.*, 1984; Orczyk *et al.*, 1987). Also Maddocks & Nelson (1952) found that man treated with hCG had high levels of urinary oestradiol which is associated with the increase in the number of Leydig cells. In contrast, a study by Siegel & Prentice (1987) using CU 32-085 did not show any significant change in circulating levels of oestradiol in the treated rats. Also Teerds *et al.* (1991) demonstrated that the appearance of Leydig cell tumours in the Wistar U-rats was not associated with any change in the circulating levels of oestradiol. Furthermore, treatment with oestrogens was found to prevent the development of Leydig cell tumours in Fischer rats (Bartke *et al.*, 1985). The latter observation was attributed to the increase in PRL levels as a result of hyperplasia of the lactotrophs (Bartke *et al.*, 1985). This prevented the increase in the levels of LH which

cause Leydig cell hyperplasia.

Considering all the conflicting results reported with respect to the correlation between the circulating levels of oestradiol and the development of Leydig cell tumours, the local levels of oestradiol produced within the testis are likely to be more important in the induction of Leydig cell tumours than the circulating levels. Since only 24% of circulating oestradiol is of testicular origin (Payne *et al.*, 1976; Lipsett, 1980), plasma levels of this steroid do not reflect the local levels in the testis which determines the incidence of the development of Leydig cell tumours. Selective sampling of the spermatic vein of a testis containing a Leydig cell tumour(s) revealed high oestrogen levels compared with the circulating levels (Gabrilove *et al.*, 1975; Castle & Richardson, 1986; Corrie *et al.*, 1987). Studies related to the implication of oestradiol in the aetiology of human breast cancer (O'Neil *et al.*, 1980; McNeill *et al.*, 1986; O'Neil & Miller, 1987; Miller & O'Neil, 1987; LØnning *et al.*, 1990) and prostatic cancer (Seppelt, 1978; Ghanadian & Puah, 1981; Iqubal *et al.*, 1983; Stone *et al.*, 1986) are in favour of the importance of the local concentrations rather than the circulating levels of oestradiol in the development of the tumours. Castle & Richardson (1986) suggested that the pathophysiology of the development of Leydig cell tumours is based on a primary Leydig cell abnormality of ostrogen production which leads to the sequential development of Leydig cell hyperplasia and eventually tumour formation.

The increase in aromatase activity and thus oestradiol levels in the Leydig cells isolated from the rats treated for 5 weeks with CU 32-085 is more likely

to be the cause for the decrease in testosterone production by those cells as discussed in Chapter 4. This is supported by studies which demonstrated that oestradiol, which is implicated in the development of Leydig cell tumours, causes a decrease in the activity of the steroidogenic enzymes (17α-hydroxylase and C17-20 lyase) involved in testosterone biosynthesis (Jones *et al.*, 1978; Forest *et al.*, 1979; Bercovici *et al.*, 1981; Mineur *et al.*, 1987). In addition, deficiency of the enzymes involved in testosterone biosynthesis have been described in Leydig cell tumours from a patient with feminizing Leydig cell tumours (Bercovici *et al.*, 1981) and in Fischer rats, with spontaneous Leydig cell tumours (Amador *et al.*, 1985; Bartke *et al.*, 1985). The change in intratesticular oestrogen/androgen levels may lead to a disturbed regulation of Leydig cell growth by altering the secretion of the testicular factors involved in this process. The involution of the seminiferous tubules in testes with Leydig cell hyperplasia, which was observed in the present study and also reported by other investigators (Brown *et al.*, 1979; Turek & Desjardins, 1979; Bercovici *et al.*, 1981; Bartke *et al.*, 1985; Mineur *et al.*, 1987) is suggestive of the involvement of local factors in the process of development of Leydig cell tumours. Considering the interations between the different types of cells within the testis (see Sharpe, 1990; Skinner, 1991 for review); it is not known if the mechanism by which this occurs is due to a lack of a controlling influence on interstitial cells by a nonfunctioning seminiferous tubule or whether damaged tubules produce an interstitial cell growth stimulator. There is evidence that Sertoli cells secrete factor(s) that stimulate Leydig cell

proliferation (Ojeifo *et al.*, 1990). In addition, since intact seminiferous tubules secrete a factor which inhibits aromatase activity (Sharpe, 1983), tubular involution could contribute to the increase in Leydig cell aromatase activity and thus local oestradiol levels which is involved in the development of Leydig cell tumours. Oestrogens have been implicated in enhancing both growth factor formation and hormonal receptor levels in endocrine target tissue (Beck & Garner, 1989), and such growth factor production via a paracrine mechanism may be involved in Leydig cell tumour aetiology. Oestrodiol induces expression of growth-related c-fos and c-myc proto-oncogenes (van der Burg *et al.*, 1989; Beck & Garner, 1989) and the c-myc oncoprotein was found to be associated with the development of testicular tumours (Sikora *et al.*, 1985; Watson *et al.*, 1986).

The possible involvement of inhibin in tumour development is supported by many observations. Serum inhibin levels were found to be abnormally elevated in patients with granulosa cell tumours (Loppöhn *et al.*, 1989) and in a post-menauposal women with a virilizing Sertoli-Leydig cell tumour (Ohashi *et al.*, 1990). Also, human Leydig cell tumours were found to produce immunoreactive inhibin (de Jong *et al.*, 1990). In the present study inhibin immunoreactivity was detected in normal and nodular Leydig cells. Based on this observation, no conclusion can be made with regard to the involvement of inhibin in the development of Leydig cell tumours caused by the DA. Further quantitative studies involving the measurement of inhibin levels in the testis interstitial fluid and in the plasma as well as its production by the isolated Leydig cells

are needed. However, a study by Teerds *et al.* (1991) showed that the development of Leydig cell tumours with ageing in Wistar U-rats was not associated with any change in circulating levels of immunoreactive inhibin. This can be due to the fact that peripheral levels of inhibin do not reflect the levels within the testis. Thus any change in the local levels of this peptide cannot be detected if measured in the periphery.

Macrophages may also play a role in the development of Leydig cell tumours. Although macrophages have often been observed in histological sections of certain types of human tumours and have been claimed to indicate good prognosis, their role in the development of tumours is still not understood. Large numbers of macrophages were found in benign and malignant breast tissue (Kelly *et al.*, 1988). In the present study an increase in the number of macrophages was found in testes from the animals treated with the DA for 57 weeks. In the testis sections where nodules of Leydig cells were found, the macrophages were always located outside the nodules. The increase in the number of macrophages as a result of treatment with the DA could be due to the sustained increase in LH caused by this treatment. This is supported by a study by Raburn *et al.* (1991) which demonstrated that treatment *in vivo* with hCG causes an increase in the number of macrophages in neonatal rat testis. The role of these macrophages is probably to prevent tumour dissemination as suggested by Eccles & Alexander (1974) and Fidler (1976). However, although the number of macrophages had increased, their cytotoxic activity may be decreased as a result of the treatment with the DA. There

is evidence that TNF, a factor secreted by macrophages and involved in their cytotoxic effect decreases with age in rats and this was found to be associated with the spontaneous occurrence of Leydig cell tumours (Bradley *et al.*, 1989; Davila *et al.*, 1990). The decrease in the production of this factor by macrophages was attributed to the decrease in circulating growth hormone (GH) levels which occur with ageing (Rudman *et al.*, 1981). This is supported by a study by Edwards III *et al.* (1991) which demonstrated that GH increases the synthesis of TNFα by macrophages, an effect mediated by an increase in the synthesis and secretion of interferon-gamma (INF-gamma) by T-lymphocytes or natural killer cells. A similar decrease in TNF levels had probably occurred in the present study and contributed to the development of the nodules of Leydig cells, since a decrease in GH was found in the rats treated with the DA for more than 4 weeks.

Based on these findings, those discussed in chapter 4, and the reported observations which support the concept of a causal relationship between the increase in LH and ACTH/glucocorticoids and the decrease in PRL and GH levels and the development of Leydig cell tumours, it can be suggested that the effect of the DA on Leydig cell growth in mediated by the changes in circulating levels of these hormones. Therefore, the decrease in testosterone production and the increase in aromatase activity in Leydig cells isolated from the rats treated with the DA for 5 weeks, found in the present study may be the cause for the development of Leydig cell tumours observed in the aged animals after long-term treatment with the DA.

CHAPTER 6

EFFECTS OF HUMAN CHORIONIC GONADOTRPHIN AND DEXAMETHASONE ON LEYDIG CELL FUNCTIONS

6.1. SUMMARY

Since treatment with the dopamine agonist (DA) (CU 32-085) results in an increase in circulating levels of LH and corticosterone, the involvement of these two hormones in the process of Leydig cell desensitization caused by DA treatment was investigated.

Sprague Dawley rats aged 8 to 10 weeks were given the vehicle (saline), hCG (100IU per rat), dexamethasone (1mg/kg body weight), or both hCG and dexamethasone. The latter group was treated with dexamethasone one hour prior to hCG. The rats were treated either once and killed 6h thereafter (acute) or treated once per day for one week and sacrified 24h after the last injection (chronic).

Both acute and chronic treatment with hCG and/or dexamethasone resulted in inhibition of LH-stimulated testosterone production. The acute treatment with hCG alone or with dexamethasone resulted in a complete desensitization of the Leydig cells with respect to testosterone and cAMP production. Pretreatment with dexamethasone prevented the increase in basal production of testosterone and cAMP caused by treatment with hCG *in vivo*. The acute treatment with dexamethasone alone had no significant effect on the basal, but inhibited the responsiveness of the cells to LH *in vitro* with respect to testosterone and cAMP production. However, the decrease in testosterone production by the cells isolated from the rats treated with dexamethasone *in vivo*, in response to the highest concentration of LH (3.3nmol/l) *in vitro*, was not associated with any significant change in cAMP formation. This suggests that the effect

of dexamethasone involved other transducing system(s) in addition to cAMP. This is supported by the finding that the acute treatment with hCG caused a marked increase in the production of $PGF_{2\alpha}$ which was inhibited by dexamethasone indicating that the effect of the latter is exerted at least partly at the level of PLA_2. The acute treatment with hCG and/or dexamethasone had no significant effect on aromatase activity or the binding of ^{125}I-labelled-hCG to the Leydig cells.

On the other hand the desensitization of the Leydig cells as a result of chronic treatment with hCG and/or dexamethasone was associated with a decrease in the binding of ^{125}I-labelled-hCG to the Leydig cells. This was reflected by the changes in peripheral levels of this androgen, since the weight of the seminal vesicles also increased in those animals.

Addition of dexamethasone *in vitro* caused a decrease in LH- and dcAMP-stimulated testosterone production in the control rats and rats treated *in vivo* with the dopamine agonist (DA) (CU 32-085) for 5 weeks. The decrease in the responsiveness of the Leydig cells from the control group as a result of treatment *in vitro* with dexamethasone was similar to that caused by treatment with the DA *in vivo*. This and the *in vivo* study with dexamethasone suggests that the effect of the DA was at least partly mediated by the increase in glucocorticoids caused by this treatment. In addition, based on the *in vivo* studies with hCG, the increase in circulating levels of LH caused by treatment with the DA may also be involved in the desensitization of the Leydig cells caused by the DA.

6.2. INTRODUCTION

Leydig cell steroidogenesis is mainly regulated by LH/hCG (Dufau *et al.*, 1971; Purvis *et al.*, 1981; van der Molen & Rommerts, 1981; Ewing *et al.*, 1983). The effect of hCG on Leydig cell function is well documented (Sharpe, 1976; Cigorraga *et al.*, 1978; Chasalow *et al.*, 1979; Dufau *et al.*, 1979; Freeman & Ascoli, 1981). Chronic treatment with LH/hCG *in vivo* was found to result in a decrease in circulating and testicular testosterone levels and in Leydig cell testosterone and cAMP production in response to LH/hCG *in vivo* (Cigorraga *et al.*, 1978; Chasalow *et al.*, 1979). This treatment was also found to cause a loss in LH receptors without affecting their affinity to LH/hCG (Sharpe, 1976; Freeman & Ascoli, 1981). Although down regulation of the LH receptors and the desensitization of the Leydig cells is believed to be specifically regulated by LH/hCG (Haour & Saez, 1977; Hsueh *et al.*, 1976, 1977; Sharpe, 1976), excess of other circulating hormones may also contribute to this process. This is supported by many investigations which demonstrated that under a range of physiological and pathological conditions, excess cortisol/corticosterone inhibited testicular function (Saez *et al.*, 1977; Mann *et al.*, 1987). This inhibitory effect can result from the effects of the glucocorticoids at three levels of the hypothalamic-pituitary-gonadal axis: the brain (to inhibit GnRH secretion) (Smith *et al.*, 1971; Belhadj *et al.*, 1989), the pituitary (to interfere with GnRH-induced LH release) (Padmanabhan *et al.*, 1983; Brann *et al.*, 1990; Nangalama & Moberg, 1991; Rosen *et al.*, 1991), and the gonads (to alter the stimulatory effect of gonadotrophins on sex steroid

secretion) (Desjardins & Ewing, 1971; Saez *et al.*, 1977; Bambino & Hseuh, 1981). In addition, an effect through inhibition of PRL release may also be involved in the inhibitory effect of glucocorticoids on Leydig cell function, since dexamethasone was found to cause a decrease in PRL release in female rats (Brann *et al.*, 1990) and PRL is known to be important in the regulation of Leydig cell function (Zipf *et al.*, 1978; Klemcke *et al.*, 1990).

The direct effect of glucocorticoids on the Leydig cells may involve inhibition of PLA_2 which is necessary for the release of arachidonic acid from phospholipids, since this enzyme is known to be inhibited by these steroids (Flower, 1988). In addition, PLA_2 was found to have a stimulatory effect on Leydig cell steroid production (Abayasekara *et al.*, 1990) and the release of arachidonic acid (Cooke *et al.*, 1991) and its effect on steroidogenesis, either directly (Lopez Ruiz *et al.*, 1991) or through its metabolites (Dix *et al.*, 1984; Didokar & Sundaram, 1987; Sullivan *et al.*, 1988; Majercik & Puett, 1991), have been demonstrated in rat Leydig cells. Also glucocorticoids have been demonstrated to down regulate LH receptors (Saez *et al.*, 1977; Bambino & Hsueh, 1981) and to exert a direct inhibitory effect on the steroidogenic enzymes, mainly, 17α-hydroxylase (Welsh *et al.*, 1982) and $P450_{scc}$ (Hale & Payne, 1989).

As described in Chapter 4, treatment with the dopamine agonist (DA) (CU 32 085) resulted in desensitization of the Leydig cells. Since the same treatment was previously found to result in an increase in circulating levels of LH and corticosterone (Siegel & Prentice, 1987), it was of interest to

investigate further the involvement of the two hormones in the process of Leydig cell desensitization caused by treatment with the DA. The effects of treatment *in vivo* with hCG and/or dexamethasone for 6h or one week on Leydig cell function was assessed using elutriated/Percoll-purified Leydig cells. The functional parameters measured include LH receptors, cAMP, testosterone, and prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) production, and aromatase activity. The effect of dexamethasone on Leydig cell steroidogenesis *in vitro* was also investigated.

6.3. RESULTS

6.3.1. Effect of treatment *in vivo* with hCG and/or dexamethasone for 1 week on body weight, and testicular and seminal vesicle weight

Chronic treatment with hCG suppressed the normal increase in body weight ($P<0.05$) (Table A3.1), had no significant effect on testicular weight (Figure A3.1a), but caused an increase in the weight of the seminal vesicles ($P<0.001$) (Figure A3.1), indicating an increase in circulating androgen levels. Chronic treatment with dexamethasone (Dex) had no significant effect on the weight of the testes and the seminal vesicles (Figure A3.1), but caused a significant decrease in body weight ($P<0.001$) (Table A3.1). The rats treated with both dexamethasone and hCG exhibited a significant decrease in body weight ($P<0.001$) similar to the group given dexamethasone alone (Table A3.1), no significant change in the weight of the testes (Figure A3.1a), and a significant increase in the weight of the seminal vesicles ($P<0.001$) similar to the group given hCG alone (Figure A3.1b).

6.3.2. Effect of treatment *in vivo* with hCG and/or dexamethasone on LH receptors and steroidogenesis in elutriated/ Percoll-purified Leydig cells

LH receptor studies, production of testosterone and cAMP, and aromatase activity were determined using elutriated/Percoll-purified Leydig cells from testes of control rats and rats treated with hCG and/or dexamethasone for 6h or one week.

6.3.2.1. Specific binding of ^{125}I-labelled hCG to Leydig cells

As shown in Figure 6.1, acute treatment with hCG and/or dexamethasone (Dex) *in vivo* for 6h had no significant effect on the specific binding of ^{125}I-labelled hCG to Leydig cells. However, chronic treatment with hCG and/or dexamethasone caused a significant decrease in the specific binding of ^{125}I-labelled hCG to the Leydig cells, with the effect being more pronounced in the group treated with hCG alone and that treated with both hCG and dexamethasone compared with the group treated with dexamethasone alone.

6.3.2.2. Testosterone and cAMP production

As shown in Figure 6.2., both acute (a) and chronic (b) treatment with hCG, and/or dexamethasone resulted in inhibition of LH-stimulated testosterone production. Acute treatment with hCG alone resulted in a complete desensitization of the Leydig cells to further stimulation with LH and basal production of testosterone (a) and cAMP (c) was higher compared with the control group. This was inhibited when the animals were pretreated *in vivo* with dexamethasone. The Leydig cells from the animals

given both hCG and dexamethasone showed a small but significant response to LH with respect to cAMP production without any parallel increase in testosterone production. Acute treatment with dexamethasone alone resulted in a decrease in the responsiveness of the Leydig cells to LH with respect to testosterone production. A parallel decrease in cAMP production was found in the cells stimulated with 0.0033, 0.033nmol LH/l but not those stimulated with 3.3 nmol LH/l.

Chronic treatment with hCG and/or dexamethasone (b) caused a decrease in the response of the Leydig cells to LH or hCG. The Leydig cells from the rats treated with hCG and those given both dexamethasone and hCG produced higher basal levels of testosterone compared with the levels produced by the cells from the controls and the group treated with dexamethasone. The Leydig cells from the rats treated with hCG or dexamethasone exhibited a small, but significant response to 0.33 nmol LH/l and 0.27 nmol hCG/l, but not to 0.0033 nmol LH/l in hCG- and dexamethasone-treated groups respectively. However, the Leydig cells from the group given both hCG and dexamethasone were not responsive to LH or hCG, but the maximum production of testosterone was not significantly different from that of the cells from the dexamethasone treated group stimulated with LH or hCG *in vitro*.

The difference in the responsiveness of the cells from the different groups cannot be attributed to a difference in the viability of the cells or macrophage content as shown in Table A3.2 and Table A3.3 respectively.

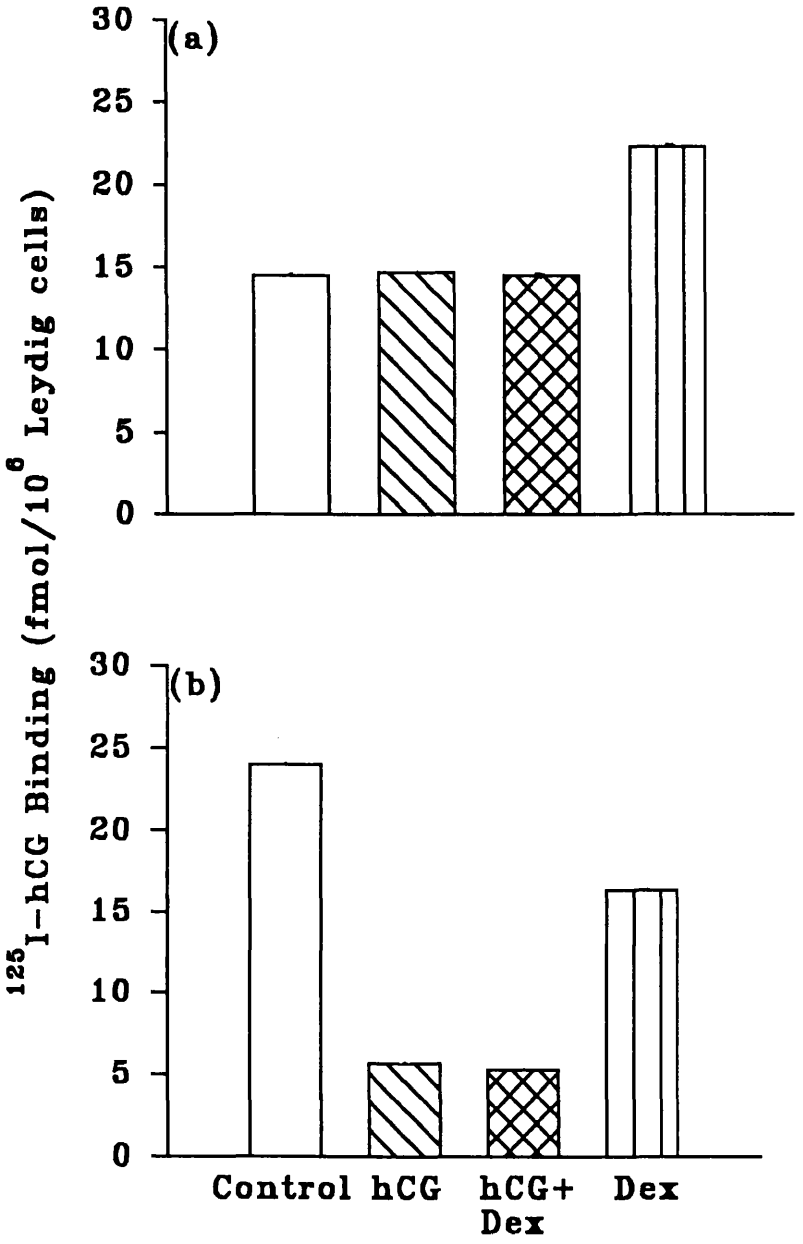
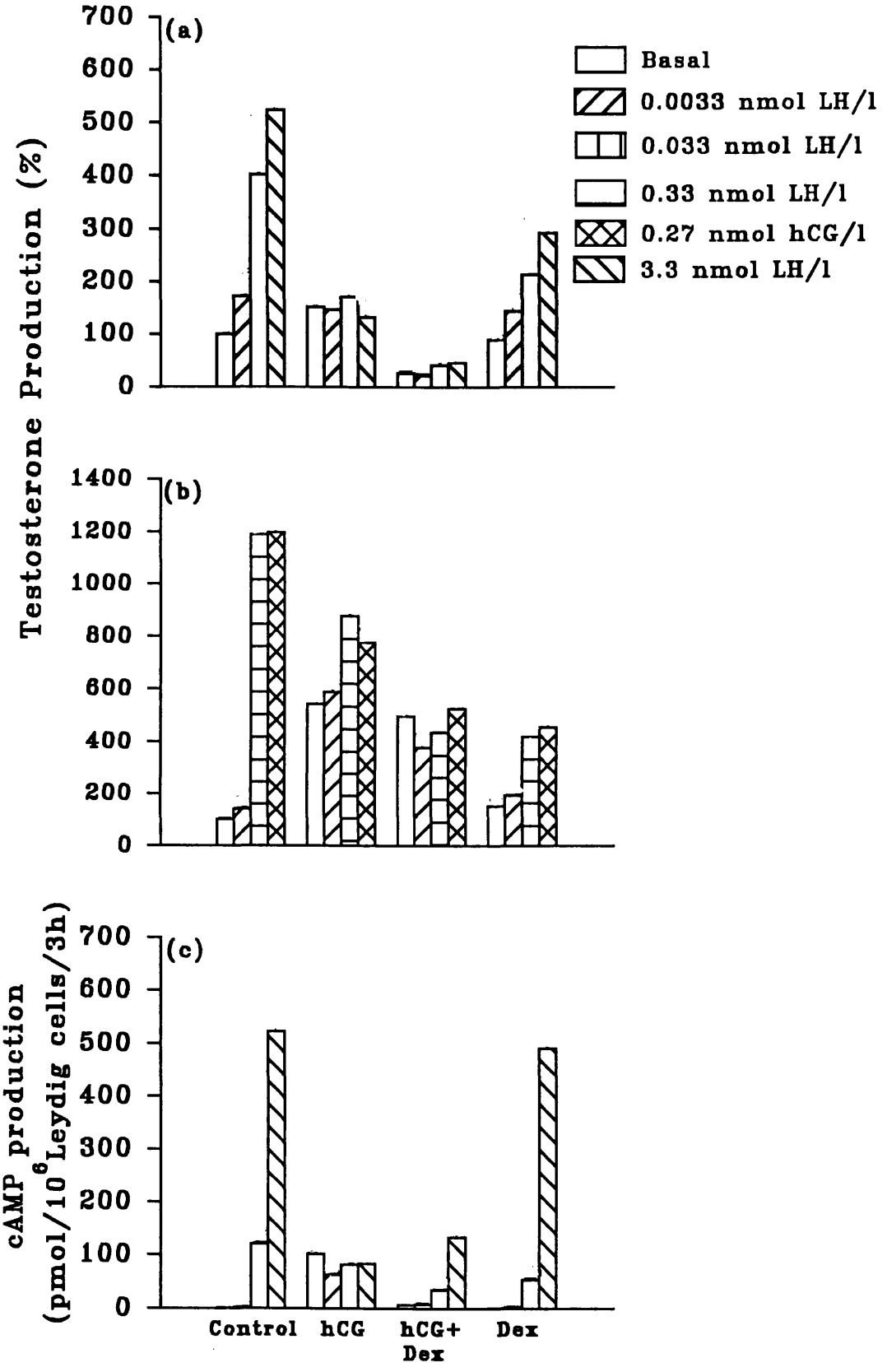


Figure 6.1. Specific binding of ^{125}I-labelled hCG to Leydig cells from testes of control rats and rats treated with hCG and/or dexamethasone. ^{125}I-Labelled hCG binding in elutriated/Percoll-purified testicular interstitial cells was determined by incubating purified cells obtained from control rats and rats treated with hCG and/or Dexamethasone (Dex) for 6h (a) or one week (b). The binding of ^{125}I-labelled hCG to Leydig cells was determined as described in Materials and Methods (Chapter 2) and expressed in fmol/10^6 Leydig cells. Results are presented as means of pooled data from two experiments each performed in triplicate.

Figure 6.2. Testosterone (a,b) and cAMP (c) production in elutriated/Percoll-purified Leydig cells from testes of control rats and rats treated with hCG and/or dexamethasone.

Production of testosterone by Leydig cells was determined in cells (10^5 cells per well) obtained from control rats and rats treated with hCG and/or dexamethasone (Dex) for 6h (a) or one week (b). The cells were incubated in 24 Costar well plates in the absence or presence of 0.0033, 0.033, 0.33, or 3.3 nmol LH/l or 0.27 nmol hCG/l for 3h. Production of cAMP was assessed in Leydig cells (10^5) isolated from testes of controls and rats treated with hCG and/or Dex for 6h (c). The cells were incubated in the absence or presence of 0.0033, 0.033, or 3.3 nmol LH/l and an inhibitor of cAMP metabolism, 0.05 mmol IBMX, was present throughout the preincubation and the incubation periods. Testosterone results were normalized by considering the basal production of this steroid by the cells from the control group (26.3 ± 5.23 (a) and 38 ± 10.7 (b) pmol/10^6 cells/3h) as 100% and cAMP production is expressed in pmol/10^6 cells/2h. Results are presented as means of pooled data from two different experiments each performed in triplicate.


(a)
(b)
(c)
Testosterone Production (%)
cAMP production (pmol/10^6 Leydig cells/3h)
Basal
0.0033 nmol LH/l
0.033 nmol LH/l
0.33 nmol LH/l
0.27 nmol hCG/l
3.3 nmol LH/l
Control
hCG
hCG+ Dex
Dex

6.3.2.3. Aromatase activity

Treatment with hCG and/or dexamethasone for 6h did not have any significant effect on aromatase activity in elutriated/Percoll-purified Leydig cells. The aromatase activity of the Leydig cells from all the groups was inhibited *in vitro* by the addition of the aromatase inhibitor, 4-hydroxy-androstenedione (4OHA).

6.3.3. Effect of treatment *in vivo* with hCG and/or dexamethasone for 6h on $PGF_{2\alpha}$ production

In order to determine the effectiveness of dexamethasone in inhibiting the PLA_2, $PGF_{2\alpha}$ was measured. It was found that hCG caused a marked increase in $PGF_{2\alpha}$ levels which was inhibited by dexamethasone. Dexamethasone had no significant effect on the basal levels of $PGF_{2\alpha}$. The difference in the production of $PGF_{2\alpha}$ by the Leydig cells from the different groups cannot be attributed to contamination with macrophages, since there is no significant difference in the macrophage content as shown in Table A8.

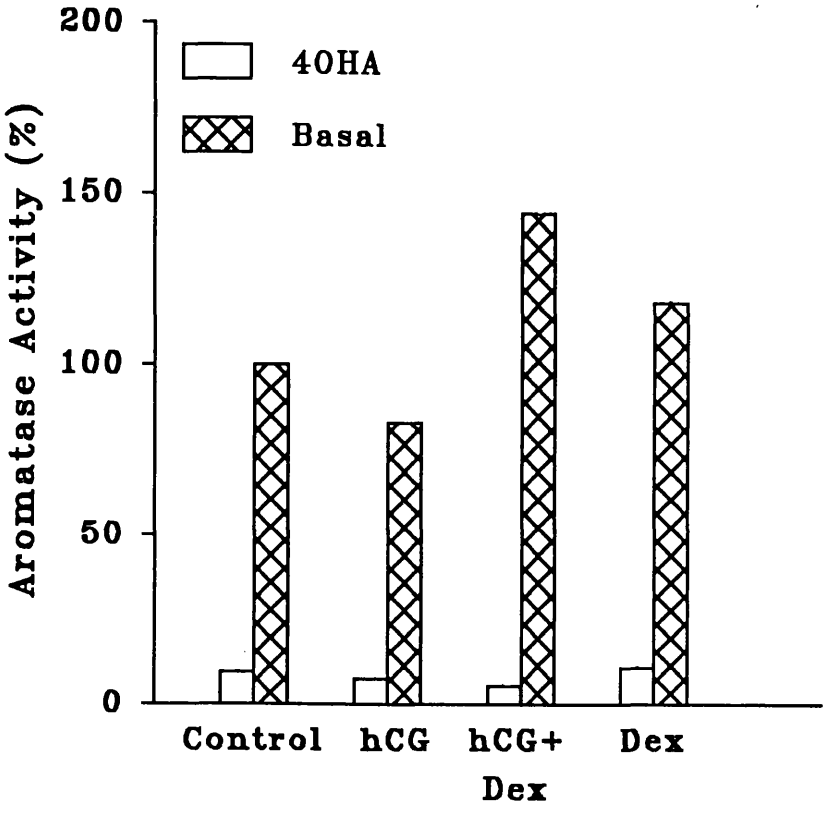


Figure 6.3. Aromatase activity in elutriated/Percoll-purified testicular interstitial cells from testes of control rats and rats treated with hCG and/or dexamethasone. Aromatase activity was assessed by measuring ^3H_2O resulting from the aromatization of [1,2,6,7-^{3}H]-androstendione as described in Figure 4.5. Aromatase activity was normalized by considering basal aromatse activity in cells from the controls (12556 dpm/10^6 Leydig cells/2h) as 100%. Results are presented as means of pooled data from two experiments each performed in triplicate.

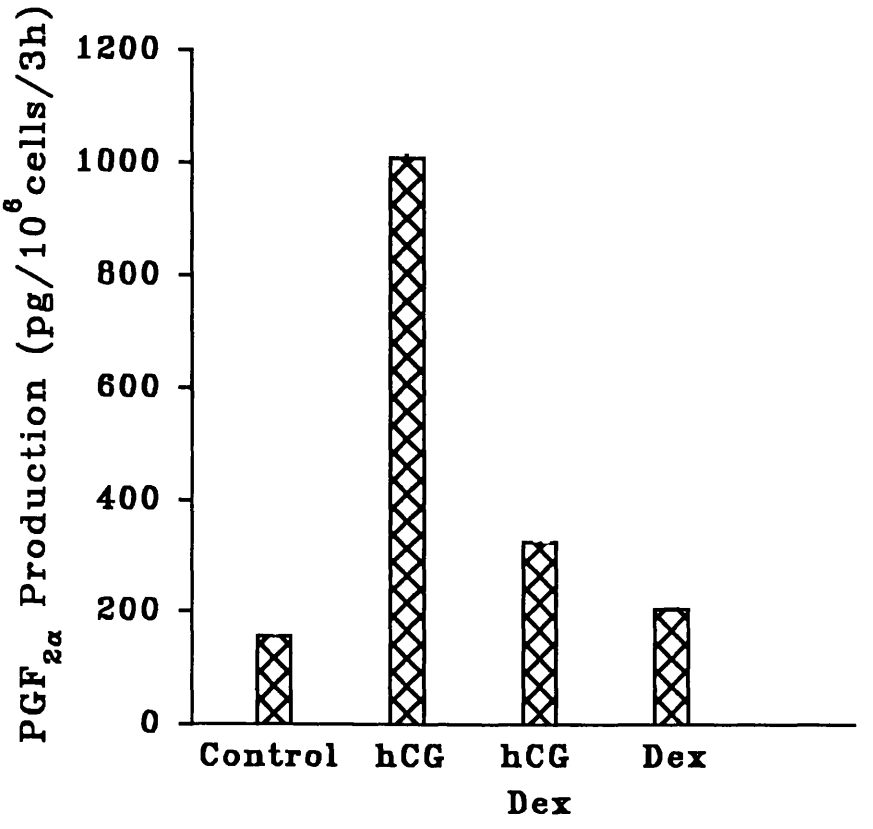


Figure 6.4. The effects of hCG and dexamethasone *in vivo* on $PGF_{2\alpha}$ production by Leydig cells *in vitro*. $PGF_{2\alpha}$ production was determined using elutriated/ Percoll purified Leydig cells isolated from the control rats and rats treated with hCG and/or dexamethasone (Dex). The purified cells were incubated for 3h as described in Materials and Methods. The medium was then collected and analysed for $PGF_{2\alpha}$. Results are presented as means of triplicate incubations.

6.3.4. Effect of addition of dexamethasone *in vitro* on the responsiveness of the Leydig cells isolated from control rats and rats treated with the dopamine agonist (CU 32-085)

Dexamethasone (25 μmol/l) caused a significant decrease in LH- and dcAMP-stimulated testosterone production in the control rats and rats treated *in vivo* with the dopamine agonist (DA) for 5 weeks. The responsiveness of the cells isolated from the rats treated *in vivo*, with the DA was similar to that of the cells isolated from the control rats and those treated with dexamethasone *in vitro*. This suggests that glucocorticoids were involved at least partly in the desensitization of the Leydig cells caused by treatment *in vivo* with the DA.

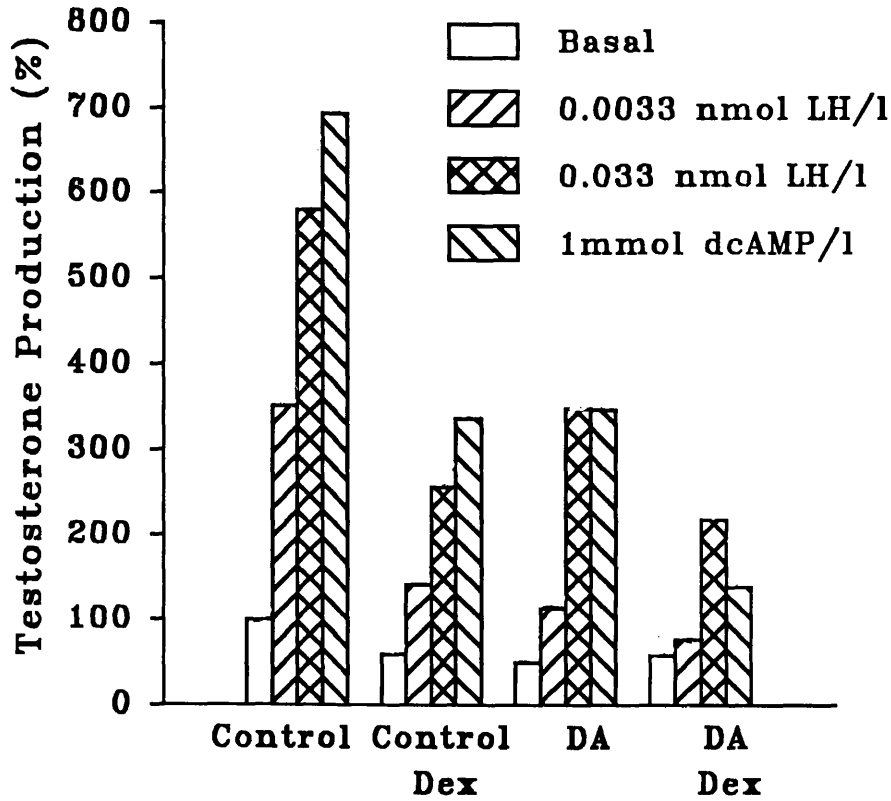


Figure 6.5. Effect of dexamethasone *in vitro* on the responsiveness of the Leydig cells isolated from control rats and rats treated with the dopamine agonist *in vivo*. Production of testosterone by Leydig cells was determined in cells (10^5 cells per well) obtained from control rats and rats treated with the dopamine agonist (CU 32 085) for 5 weeks. The cells were incubated in the absence or presence of 25µmol dexamethasone (Dex)/l for 30min. Then the cells were incubated in the absence or presence of 0.0033 or 0.033 nmol LH/l or 1mmol dcAMP/l for 2h. Results were normalized by considering the basal production of this steroid by the cells from the control group (32.4 ± 4.88 pmol/10^6 cells/2h) as 100%. Results are presented as means of pooled

6.4. DISCUSSION

The studies with hCG and dexamethasone confirmed previous studies which demonstrated that exposure to high concentrations of either compounds supressed Leydig cell function. They also support our view that the process of desensitization of the Leydig cells caused by treatment with the dopamine agonist (DA) (CU 32-085) was caused by the increase in LH and corticosterone which was found to occur after such treatment (Siegel & Prentice, 1987).

The increase in testicular $PGF_{2\alpha}$ production in response to treatment *in vivo* with hCG is in line with previous studies which showed an increase in $PGF_{2\alpha}$ production by the testes in response to treatment with hCG *in vivo* (Haour *et al.*, 1979). Furthermore, Abayasekara *et al.* (1990) have reported an increase in the levels of $PGF_{2\alpha}$ in the interstitial fluid 4-8h after a single injection of 100IU hCG. The inhibition of the production of this prostaglandin in response to hCG by pretreatment with dexamethasone, which was demonstrated for the first time in Leydig cells, is consistent with dexamethasone action being at least partly mediated via inhibition of PLA_2 (Flower *et al.*, 1988).

In agreement with previous *in vitro* and *in vivo* studies (Cigorraga *et al.*, 1978; Catt *et al.*, 1979, it was found that hCG caused desensitization of the subsequent responses to LH in terms of cAMP and testosterone production and that basal levels were increased. The acute effects of hCG were more likely due to uncoupling of the LH receptor transducing system, since the formation of cAMP in response to LH was inhibited. The chronic treatment, however, may involve the decrease in LH receptors as well as a lesion in

steroidogenesis as reported by many investigators (Chasalow *et al.*, 1979; Dufau *et al.*, 1979a; Nozu *et al.*, 1981). The latter was attributed to the inhibitory effect of intratesticular oestradiol formation upon testosterone biosynthesis (Hsueh *et al.*, 1978; Dufau *et al.*, 1979; Nozu *et al.*, 1981a,b,c, Aquilano & Dufau, 1983; Tsai-Morris *et al.*, 1985). The down regulation of the LH receptors can be due to internalization and degradation of the receptor (see Rommerts & Cooke, 1988 for a review) as well as a decrease in LH receptor mRNA levels (Pakarinen *et al.*, 1990).

The desensitization of the Leydig cells as a result of acute treatment with dexamethasone *in vivo* can be due to inhibition of PLA_2. In addition, dexamethasone exerted an inhibitory effect on cAMP production by the Leydig cells in response to 0.033 nmol LH/l. The latter observation is in agreement with the studies reported by Welsh *et al.* (1982) in which dexamethasone was found to decrease hCG-stimulated cAMP production. However, the production of cAMP in response to the highest concentration of LH (3.3 nmol/l) was not affected by the treatment with dexamethasone *in vivo.* This is in agreement with the *in vitro* studies by Abayesakara *et al.* (1990) which showed that cAMP production in response to 3.3 nmol LH/l was not affected by treatment with dexamethasone. On the other hand, the chronic treatment with dexamethasone caused desensitization of the Leydig cells associated with a decrease in the binding of ^{125}I-labelled hCG to those cells. The latter observation is in agreement with the results reported by Saez *et al.* (1977) and Bambino & Hsueh (1981). The mechanism by which dexamethasone causes a

decrease in LH receptors is not known, a direct inhibitory effect on the synthesis of the receptor may be involved in this process. In addition, this can be attributed to the gluconeogenic effect of dexamethasone (Jamal *et al.*, 1991), since there is evidence that experimental induction of diabetes in rats leads to reduction in the concentration of LH receptors in the testis (Charreau *et al.*, 1978). A decrease in PRL release as a result of treatment with dexamethasone, which has been demonstrated by Brann *et al.* (1990) in female rats, may also contribute to this effect.

In addition to inhibition of PLA_2, cAMP production, and down regulation of LH receptors, inhibition of the activity and/or the synthesis of the steroidogenic enzymes, mainly $P450_{scc}$ (Hale & Payne, 1989) and 17α-hydroxylase (Welsh *et al.*, 1982) may also be involved in the chronic effect of dexamethasone.

The higher basal levels of testosterone produced by the Leydig cells isolated from the rats treated with hCG and those given both hCG and dexamethasone compared with the controls, were reflected by higher circulating levels of this steroid; both treatments resulted in an increase in the weight of the seminal vesicles. Unlike the acute treatment, the higher basal production of testosterone and the increase in the weight of the seminal vesicles caused by chronic treatment with hCG was not inhibited by pretreatment with dexamethasone. This could be due to the fact that dexamethasone has a shorter half life compared with hCG.

The inhibitory effect exerted by dexamethasone on testosterone production in

response to LH and dcAMP, *in vitro*, confirmed previous studies by Abayesakara *et al.* (1990) which showed that dexamethasone and other inhibitors of PLA_2 caused such inhibition. This supports further the involvement of arachidonic acid and its metabolites in the regulation of Leydig cell steroidogenesis as demonstrated in previous studies (Dix *et al.*, 1984; Didokar & Sundaram, 1987; Sullivan *et al.*, 1988; Majercick & Puett, 1991). The similarity in the desensitization of the Leydig cells caused by treatment with dexamethasone *in vitro* and that which resulted from treatment with the DA *in vivo*, supports the suggestion that the effect exerted by the latter was at least partly mediated by the increase in glucocorticoids caused by this treatment. In addition, the chronic *in vivo* studies with hCG support the involvement of the increase in circulating levels of LH caused by treatment with the DA in the desensitization of the Leydig cells. The complete desensitization of the Leydig cells from the animals treated with hCG, which did not occur in the cells from the animals treated with the DA, could be due to the large dose (100IU per rat) of hCG administered to those animals compared with the levels of circulating LH resulting from treatment with the DA which were only twice the normal levels (Siegel & Prentice, 1987). Unlike treatment with hCG, the basal production of testosterone by the Leydig cells from the rats treated with the DA was either unchanged or decreased. This could be attributed to the inhibitory effect exerted by the increase in glucocorticoids which was found to occur as a result of the same treatment with the DA (Siegel & Prentice, 1987).

CHAPTER 7

GENERAL DISCUSSION

In order to investigate the effects of the dopamine agonist CU 32-085 (DA), methods have been developed in the present study, to separate and characterize different Leydig cell populations and to purify them from other testis cells including macrophages. This methodology has been applied to the Leydig cells obtained from rats treated with the DA. The results clearly indicate that changes in Leydig cell function do occur at very early stages; the nature of these changes and their relationship to tumour formation will now be discussed:

7.1. The possible mechanisms involved in the effect of the DA on Leydig cell function(s) in rats

The changes in Leydig cell function caused by treatment with the DA *in vivo* were probably mediated by the changes in circulating hormone levels (increase in LH and ACTH/ glucocorticoids, and a decrease in PRL) resulting from its effects on the central nervous system (Siegel & Prentice, 1987). Although direct inhibitory effects of the DA on Leydig cell testosterone production were shown in the present study, they are likely to be non-specific, since high concentrations ($>10^{-5}$ mol DA/l) were required. Also, in contrast to the treatment with the DA *in vivo*, this effect was not accompanied by a decrease in cAMP production. The indirect effect of the DA is further supported by a study by Marko (1984) which showed that the DA metabolites (which mediate the effects of the DA) concentrate in the striatum rather than in the plasma. In addition, the autoradiographic studies, using [^{3}H]-CU 32-085, did not reveal any detectable specific binding in the testis (Siegel & Prentice, 1987).

The decrease in the capacity of the Leydig cells to bind ^{125}I-hCG as a result of treatment with the DA can be attributed to the decrease in circulating levels of PRL and the increase in LH and ACTH/glucocorticoids. Since, LH receptors are known to be regulated by PRL (Zipf *et al.*, 1978) and chronic treatment *in vivo* with hCG (Sharpe, 1976; Freeman & Ascoli, 1981) or glucocorticoids (Saez *et al.*, 1977; Bambino & Hsueh, 1981) were found to cause a decrease in the number of LH receptors in rat testes.

The decrease in the number of LH receptors was accompanied by a decrease in the response to LH in terms of cAMP and testosterone production. In addition, treatment with the DA had a differential effect on the capacity of the cells to produce testosterone; the Leydig cells with lower sedimentation velocities produced less basal and LH-stimulated testosterone compared with their respective controls. The Leydig cells with higher sedimentation velocities, however, exhibited higher basal levels and a similar capacity to produce testosterone in response to LH compared with the corresponding controls.

The decrease in the responsiveness of the Leydig cells (desensitization) from the treated animals cannot be atributed to the decrease in LH receptors and cAMP production, since the remaining LH receptors and the levels of cAMP produced by the Leydig cells in response to LH are enough to maintain steroidogenesis (Dufau *et al.*, 1975). A possible lesion in the steroidogenic pathway was investigated and this was found to be due to a defect in the 17α-hydroxylase/C17-20 lyase. A similar lesion was found to result from treatment *in vivo* with LH/hCG (O'Shaunessy *et al.*, 1982), and this was found

to be associated with a decrease in the levels of 17α-hydroxylase and C_{17-20} lyase (Cigorraga *et al.*, 1978; Dufau *et al.*, 1979). This was attributed to testicular 17ß-oestradiol, since the steroidogenic defect was found to be preceded by an increase in Leydig cell aromatase activity (Tsai-Morris *et al.*, 1986, 1988) and increased production of oestradiol after LH/hCG treatment (Cigorraga *et al.*, 1980). In the present study, the steroidogenic defect was also found to be associated with an increase in aromatase activity, thus indicating a potential for increased oestrogen production by the Leydig cells as a result of treatment with the DA.

The increase in aromatase activity (i.e. oestradiol production) and the decrease in testosterone production in response to LH, dcAMP, or 22R-hydroxycholesterol found in the present study using isolated Leydig cells after *in vivo* treatment with the DA further strengthens the importance of using such a system rather than measuring peripheral levels of oestradiol and testosterone which did not exhibit any significant change (Siegel & Prentice, 1987).

The steroidogenic lesion observed in the present study can also be due to the increase in the levels of glucocorticoids caused by treatment *in vivo* with the DA (Siegel & Prentice, 1987), since there is evidence that treatment *in vivo* with corticosteroids causes a decrease in Leydig cell steroidogenesis (Bambino & Hsueh, 1981). This effect could be exerted through inhibition of PLA_2.

In addition to oestradiol and glucocorticoids, paracrine and/or autocrine factors may also be involved in the desensitization of the Leydig cells caused by

treatment *in vivo* with the DA. IL1ß (Calkins *et al.*, 1988), EGF (Welsh & Hsueh, 1982), and TGFß (Avallet *et al.*, 1987; Lin *et al.*, 1987) which are produced by testicular cells (see Skinner, 1991 for a review) were found to exert an inhibitory effect on Leydig cell testosterone production.

The decrease in the volume of the interstitial fluid (IF) as a result of treatment *in vivo* with the DA could be a consequence of changes in testosterone and oestradiol production. This is because chronic treatment of intact rats with 17ß-oestradiol was found to decrease testicular IF volume (Widmark *et al.*, 1987) and the maintenance of the latter was found to require testosterone and to involve the seminiferous tubules (Maddocks & Sharpe, 1989). This suggests a defect in the local regulation of testicular functions.

The studies with hCG and dexamethasone support our view that the process of desensitization of the Leydig cells caused by treatment with the dopamine agonist (CU 32-085) (DA) was at least partly caused by the increase in LH and corticosterone which occur as a result of treatment with this compound.

The desensitization of the Leydig cells caused by the acute treatment with hCG were more likely due to uncoupling of the LH receptor transducing system involving adenylate cyclase, since the formation of cAMP in response to LH was inhibited. In addition, the increase in $PGF_{2\alpha}$ caused by this treatment may also be involved in this process as suggested by Haour *et al.*, 1979. The mechanism by which $PGF_{2\alpha}$ exerts this effect is unknown, but binding sites for $PGF_{2\alpha}$ as well as for PGE_1 and PGE_2 are present on the Leydig cells (Haour *et al.*, 1979). Thus, $PGF_{2\alpha}$ originating from Leydig cells or

from other testicular cells, e.g. macrophages may be involved in the desensitization of the Leydig cells caused by treatment with LH/hCG.

The chronic treatment with hCG may involve a decrease in LH receptors as well as a lesion(s) in steroidogenesis as reported by many investigators (Nozu *et al.*, 1981). The down regulation of LH receptors can be due to internalization and degradation of the receptors (see Rommerts & Cooke for a review) as well as a decrease in the mRNA (Pakarinen *et al.*, 1990).

The desensitization of the Leydig cells as a result of acute treatment with dexamethasone *in vivo* can be due to inhibition of PLA_2 as demonstrated in the present study by the inhibition of $PGF_{2\alpha}$ production in response to hCG treatment *in vivo* and thus inhibition of production of arachidonic acid and its metabolites, 5-HETE, 12-HETE, 15HETE, and 15HPETE, which were found to be involved in the regulation of Leydig cell steroidogenesis (Sullivan *et al.*, 1988; Majercik & Puett, 1991; Lopez-Ruiz *et al.*, 1992). In addition, dexamethasone was found to interact with the cAMP pathway; inhibition of cAMP production in response to submaximal concentrations of LH occurred in the Leydig cells from the rats treated with dexamethsone *in vivo*. This effect may result from inhibition of the production of arachidonic acid, since the latter was found to interact with the other transducing systems which involve PKC and cAMP (Lin, 1985; Parker *et al.*, 1987; Platts *et al.*, 1988). The chronic treatment with dexamethasone which resulted in desensitization of the Leydig cells can be partly due to the decrease in the number of LH receptors demonstrated in the present study and others (Saez *et al.*, 1977; Bambino &

Hsueh, 1981). It can also result from the decrease in the steroidogenic enzyme, 17α-hydroxylase, as shown by Welsh *et al.* (1982).

7.2. The possible mechanisms involved in the development of Leydig cell tumours as a result of treatment with the DA in rats

Based on the studies which demonstrated that LH/hCG is implicated in Leydig cell growth in rats (Brown *et al.*, 1979; Hodgson & Hudson, 1983; Léon *et al.*, 1987) and men (Maddocks & Nelson, 1952), it can be suggested that the increase in the incidence of Leydig cell tumours in Sprague Dawley rats, caused by treatment with the dopamine agonist (CU 32-085) (DA), can be a result of chronically elevated circulating LH levels. This is supported by the fact that other structurally and pharmacologically different compounds which induce Leydig cell tumours also lead to elevated serum LH levels in the rat (Roberts *et al.*, 1989). In addition to the increase in LH, the decrease in PRL as a result of treatment with the DA *in vivo* may also be involved in the induction of Leydig cell tumours caused by treatment with the DA. This is supported by Amador *et al.* (1985) and Bartke *et al.* (1985) who suggested that the induction of Leydig cell tumours in the aged Fisher rats resulted from the decrease in circulating levels of prolactin.

The involution of the seminiferous epithelium which was found in the present study and others (Mineur *et al.*, 1987; Teerds *et al.*, 1991) to be confined to regions of the testis containing nodules of hyperplastic Leydig cells can be due to the local reduction in the availability of testosterone to the seminiferous epithelium and/or to the disruption of the local paracrine control. The

involvement of local paracrine/autocrine factors in the process of development of Leydig cell tumours is supported by a study by Ojeifo *et al.* (1990) which demonstrated that Sertoli cells stimulate Leydig cell proliferation. Oestradiol may also be involved in the induction of Leydig cell tumours. In the present study, the increase in aromatase activity (i.e. oestradiol production) which was found to occur in the Leydig cells from the rats treated with the DA for 5 weeks was not associated with any increase in the number of Leydig cells. However, a hypertrophic effect of the DA cannot be excluded since the Leydig cells from the treated animals showed an increase in the rate of protein synthesis and also a shift in Leydig cell distribution when separated on the basis of their sedimentation velocities. Thus, the increase in aromatase activity at an early stage of treatment is more likely involved in the induction of Leydig cell tumours which appear at a later age. Although the correlation between the incidence of Leydig cell tumours and circulating levels of oestradiol is still not conclusive, many observations support the implication of the local levels of oestradiol in the induction of tumours in the testis (Castle & Richardson, 1986) or other endocrine organs including the prostate (Stone *et al.*, 1986) and breast (Miller & O'Neil, 1987). The effect of oestradiol on Leydig cell growth may involve oncogene expression, since this steroid was found to induce expression of growth related c-fos and c-myc proto-oncogene (Beck & Garner, 1989) and the c-myc oncoprotein was found to be associated with the development of testicular tumours (Watson *et al.*, 1986).

The possible involvement of inhibin in the development of Leydig cell tumours

cannot be excluded, since there is evidence that Leydig cell tumours produce immunoreactive inhibin and high circulating levels of inhibin were found in patients with granulosa cell tumours (Loppöhn *et al.*, 1989) and virilizing Sertoli-Leydig cell tumour (Ohashi *et al.*, 1990). In the present study, inhibin immunoreactivity was detected in normal and nodular Leydig cells. However, quantitative studies are needed to determine whether there is any correlation between the incidence of Leydig cell tumours and the local levels of inhibin. Macrophages may also be involved in the development of Leydig cell tumours. An increase in the number of macrophages was found in the present study in the testes from the rats treated for 57 weeks. This observation is supported by a study by Kelly *et al.* (1988) which showed the presence of large numbers of macrophages in benign and malignant breast tissue. However, although the number of macrophages had increased in response to treatment *in vivo* with with the DA, their cytotoxic activity may be decreased as a result of the treatment with the DA. There is evidence that TNF, a factor secreted by macrophages and involved in their cytotoxic effect decreases with age in rats and this was found to be associated with the spontaneous occurence of Leydig cell tumours (Davilla *et al.*, 1990). The decrease in the production of this factor by macrophages was attributed to the decrease in circulating growth hormone (GH) levels which occur with ageing (Rudman *et al.*, 1981). Since a decrease in GH was also found to occur as a result of treatment with the DA (Siegel & Prentice, 1987), a decrease in TNF can result from treatment with the DA and thus can promote the induction of Leydig cell tumours.

7.3. Safety assessment of CU 32-085 in man: is the extrapolation of the finding in the rat valid?

Since no long-term human studies with the dopamine agonist (CU 32-085) have as yet been performed, it is not known whether a similar increase in circulating LH levels, found in the rat, will occur in response to this compound in human. Based on studies with other dopamine agonists, no alterations in LH levels were observed in most cases (de Marinis *et al.*, 1983; Igorashi, 1983). However, the re-establishment of normal blood concentrations of LH from subnormal pretreatment levels has been reported after treatment with bromocriptine (Ambrosi *et al.*, 1985; Kletzy *et al.*, 1986).

The relevance of the rat tumour finding to safety assessment with CU 32-085 in man can be questioned on the basis of many differences between the two species:

1) There are well established differences in the regulation of the rat and human hypothalamic-pituitary-gonadal (HPG) axes. The hypothalamic adrenergic neurotransmission play a major role in the regulation of HPG function in the rat (Barraclough *et al.*, 1985; Kalra, 1986) but not in human. The effect of ageing upon the rat and human HPG axes is different. The hypothalamus (particularly the dopaminergic neuronal system) is especially sensitive to the ageing process in the rat (Simpkins *et al.*, 1977), while in man, age related dysfunction of the reproductive endocrine system originates at the level of the target glands (Angeli *et al.*, 1985; Fabbrini *et al.*, 1985).

2) The incidence of Leydig cell tumours in human is less than 1 per 10 million

(Lee *et al.*, 1981), while a relatively high incidence of spontaneous Leydig cell tumours occur in rats (Turek & Desjardins, 1979; Teerds *et al.*, 1991). In addition, the extreme sensitivity of rat Leydig cells to injury by a variety of agents has no parallel in the human.

3) In humans, in contrast to the rat, hyperstimulation with gonadotrophic hormones has not been found to induce consistently Leydig cell hyperplasia. In an early investigation in men with various forms of testicular dysfunction, hCG increased the number of Leydig cells (Maddock & Nelson, 1952). On the other hand, in a study in which hCG was given to normal human males for up to 16 weeks, Leydig cell hyperplasia did not occur (Heller & Leach, 1971).

In addition, chronically elevated serum LH levels in patients with gonadotrophin-secreting pituitary tumours, did not cause Leydig cell proliferation (see Siegel & Prentice for Refs).

The relevance of the rat tumour finding to safety assessment with the DA in man may be further questioned by the fact that the increase in the incidence of Leydig cell tumours occurs only in rats treated with this DA for more than half their lifetime and particularly early in life. The use of this DA in human, however, would involve relatively shorter periods of treatment and usually at a later age. However, the changes in circulating levels of glucocorticoids and PRL which result from treatment with the DA (CU 32-085) in rats raise the question of a possible tumorogenic effect of the DA in man. The decrease in PRL and the increase in glucocorticoids would probably cause changes in the local control which would lead to the development of Leydig cell tumours.

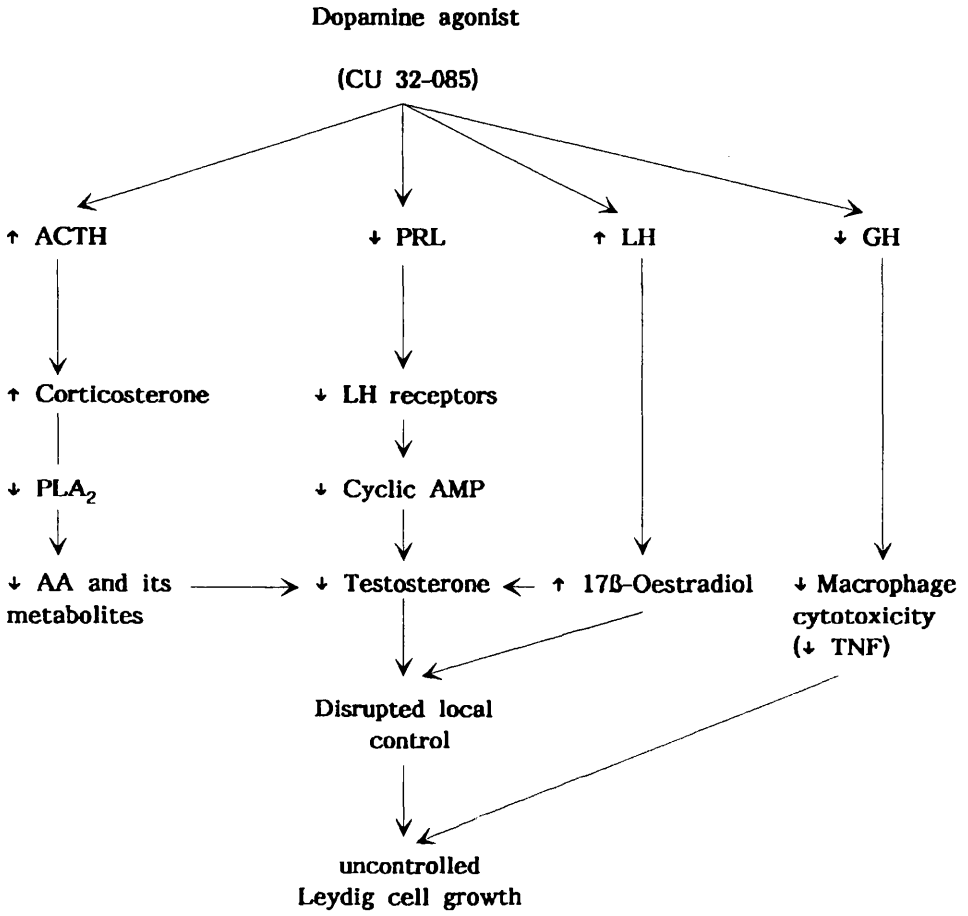


Figure 7.1. The possible mechanisms involved in the effect of the dopamine agonist (CU 32-085) on Leydig cell function(s) and growth in Sprague Dawley rats.

7.4. Development of a method for the separation of Leydig cells from macrophages and the study of Leydig cell heterogeneity

This study demonstrated the successful application of centrifugal elutriation in combination with Percoll-density gradient centrifugation and immunomagnetic particles (Dynabeads M450) coated with ED2, to obtain highly pure Leydig cell preparations. The Leydig cells obtained by using this procedure of purification retained their viability and steroidogenic activity.

Although the immunomagnetic separation of the remaining macrophages from the Leydig cells did not alter the responsiveness of the purified Leydig cells to LH, this is necessary in studies in which the interactions of macrophages with Leydig cell are investigated and also when ligands which might affect the activity of macrophages are used. This method can also be used for the positive selection of macrophages (Lea *et al.*, 1986) and this will allow the characterization of testicular macrophages and the study of their interaction with Leydig cells and other testicular cells.

In agreement with O'Shaunessy *et al.* (1981) and Chase & Payne (1983), the functional heterogeneity of the Leydig cells was observed in the present study. The Leydig cells with the highest sedimentation velocities (i.e. larger size) were more responsive to LH with respect to testosterone and cAMP production compared with those of the lowest sedimentation velocities. In contrast to the results reported by Bhalla *et al.* (1987) and Browne *et al.* (1990), the Leydig cell functional heterogeneity was not associated with any difference in the capacity of the Leydig cells to bind ^{125}I hCG. This discrepancy can be attributed

to the difference in the incubation temperature at which ^{125}I-labelled hCG binding was carried out (Habberfield *et al.*, 1987) and also to the difference in the procedure of isolation of the Leydig cells which may result in the release of different populations of Leydig cells, degree of contamination by other testicular cells, and/or cell damage. The functional heterogeneity found in the present study was not due to cell damage, nor to the contamination with macrophages, since no significant difference in the cell viability and the macrophage content was found in the fractions containing Leydig cells separated on the basis of their sedimentation velocities.

7.4. Proposals for further work

To investigate further the mechanisms involved in the induction of Leydig cell tumours by treatment with the dopamine agonist (DA) (CU 32-085), the following studies can be carried out:

1) Thymidine uptake by the Leydig cells should be determined using testicular sections to determine the origin of the hyperplastic Leydig cells. Special attention should be given to the fibroblast-like cells usually found in the regions of the testis sections containing nodules of hyperplastic Leydig cells.

2) Electron microscopy study should be carried out to determine whether the treatment with the DA result in any change in peroxisome proliferation

3) The involvement of oncogene expression (e.g. c-myc and c-fos) in the process of development of Leydig cell tumours should be considered.

4) Inhibin immuno- and bioactivity should be measured in culture medium from isolated Leydig cells and in the interstitial fluid.

5) The possible inflamatory reaction caused by DA-treatment should be investigated by measuring IL-1 activity in condition medium of purified Leydig cells, purified testicular macrophages, and in testicular IF.

6) The mechanism(s) for the decrease in the IF volume as a result of treatment with the DA should be further investigated.

7) The involvement of changes in local factors in the induction of Leydig cell tumours should be determined. Some of functional parameters for Sertoli cells and germ cells (e.g. production of certain specific proteins which may be mitogenic) should be studied to find out whether the treatment with the DA will result in any change in their production. Also, the possible involvement of factors secreted by Sertoli cells and/or germ cells in the process of Leydig cell proliferation should be investigated.

8) The levels and the localization of oestrogen receptors should be determined to see whether this correlates with the induction of Leydig cell tumours.

9) The lesion in steroidogenesis which was found to result from a defect in the 17α-hydroxylase and 17-20 lyase should be further investigated to find out whether both or either of the two enzymes were affectd by the treatment with the DA. This can be carried out by using progesterone or 17α-hydroxyprogesterone and measuring the production of 17α-hydroxyprogesterone and testosterone respectively.

10) The increase in aromatase activity in the DA-treated rats should be further investigated by measuring oestradiol production in the interstitial fluid, and testicular venous blood. *In situ* hybridization for aromatase is also needed

to determine whether other testicular cells (Sertoli cells), in addition to Leydig cells, contribute to the increase in the local production of oestradiol.

Aromatase activity (i.e. oestradiol levels) should be determined in the Leydig cell populations separated on the basis of their sedimentation velocities to determine whether the differential effect of the DA on testosterone production is associated with a similar effect on this enzyme.

11) Further studies to determine the exact source for the increase in $PGF_{2\alpha}$ levels in the IF, which occur after treatment with hCG *in vivo*. This will involve measuring $PGF_{2\alpha}$ in the interstitial fluid and also in isolated Leydig cells and testicular macrophages. The latter can be purified either by immunomagnetic separation using Dynabeads M450 coated with the macrophage monoclonal antibody (ED2) or using a fluorescent cell sorter.

The possible involvement of $PGF_{2\alpha}$ in the desensitization of the Leydig cells should be determined. This will involve preincubation of the cells with $PGF_{2\alpha}$ and then stimulation with LH.

APPENDIX 1: METHODS

Appendix 1.1. Media used for collagenase diggestion, elutriation, Percoll-purification and primary cultures

1) Dulbecco's modified Eagle's medium (DMEM) (pH 7.4) (1l)
DMEM (100ml concentrated solution (10x) or an equivalent amount in a powdered form (13.4g)) was substituted with 0.5g streptomycin, 250 x 10^3 units penicillin, and 1g BSA. This was made up to 1l with water and adjusted to pH 7.4 with concentrated NaOH.
This medium was used for washing the testes before dissection and for primary cultures in an air incubator. $NaHCO_3$ (3.7g/l) was added when incubations were carried out in 95% air/ 5% CO_2.

2) Digestion medium (40ml)
DMEM (medium 1) pH 7.4
20mg collagenase
8mg soybean trypsin inhibitor (860µl of 1% sterile sulution)

3) Elutriation medium (pH 7.4) (1l)
DMEM (same as in medium 1) substituted with 0.5g streptomycin, 250 x 10^3 units penicillin, 1mmol EDTA, 10mM Hepes, and 5g BSA. This was made up in medium 1.

4) Preparation of 0-90% continuous Percoll gradients
90% Percoll solution (25ml)
2.5ml of Earles balanced salt solution (EBSS) (x10)
0.25ml of 1M Hepes (adjusted to pH 7.4 with concentrated NaOH)
0.25ml streptomycin/penicillin solution containing 6250IU penicillin and 18.5mg streptomycin.
This was made up to 25ml with Percoll solution. The continuous Percoll density gradient 0-90% was prepared by pumping 25 ml DMEM (medium 1) into a 90% Percoll solution using a Gilson pump (Gilson minipuls 2, Villiers. Le.Bel, France) set at 280 (flow rate= 2.25ml/min) with tubing of 2.5mm diameter. As the first drop of dissection medium enters the 90% Percoll solution, the latter was pumped into a 2 x 25ml universals.

BSA was always added at the end and was dissolved by gentle stirring using a magnetic stirrer to avoid frothing. Medium 1 and 3 were sterilised by filtering through a 0.2 µm AcroCap filter (AcroCap™, Gelman Sciences Inc, Michigan, USA) using a Millipore pump (model 17015.72) and stored at 4°C until used.

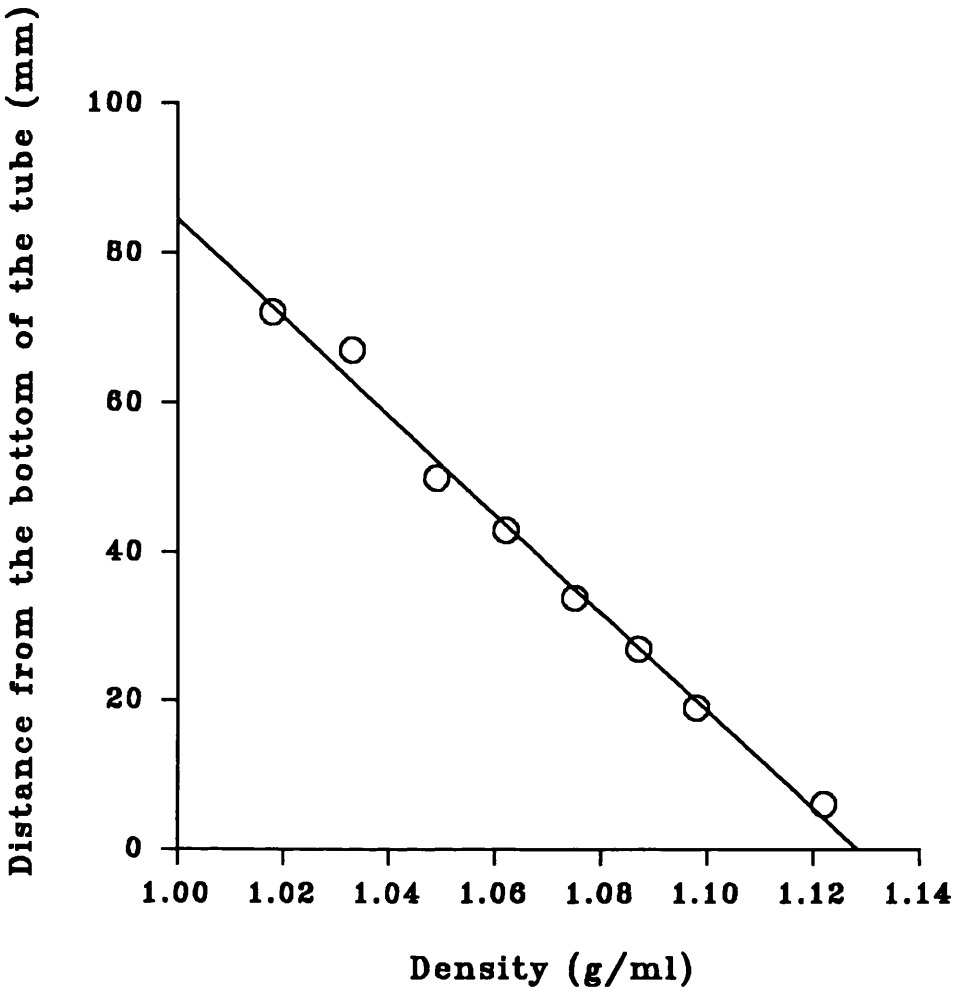


Figure A1.1. Typical standard Percoll density gradient profile.
Freezed-dried couloured density marker beads, corresponding to densities (1.018, 1.033, 1.051, 1.064, 1.076, 1.087, 1.098, and 1.120 g/ml), were swollen in water, then washed an resuspended in buffered saline before use. The marker beads were added in a 10 µl volume on the top of a 0-90% linear Percoll gradient. The volume of the gradients was adjusted with DMEM to match the volume of those containing the interstitial cells. The gradients were centrifuged at 1300g for 25min, then the position of the marker beads was marked and their distance from the bottom of the gradients (24ml universal) was measured. Results represents mean ± SD of 4 different experiments.

Fraction number	Density (g/ml)
F1	1.060 ± 0.013
F2	1.057 ± 0.021
F3	1.057 ± 0.011
F4	1.077 ± 0.017
F5	1.072 ± 0.006
F6	1.074 ± 0.016
F7	1.067 ± 0.013
F8	1.080 ± 0.002

Table A1.1. Leydig cell densities after elutriation/Percoll purification (band 2). The densities of the Leydig cells from fraction F1 to F8 were determined by adding the interstitial cells from each fraction, which were resuspended in 2ml DMEM, on the top of a 0-90% linear Percoll gradient. Density marker beads were added to one or two separate gradients as described in Figure A1 and DMEM was added to adjust the volume of those gradients. All gradients were then centrifuged at the same time at 1300g for 25min. The cell bands were marked as well as the the marker beads and the distance from the bottom of the gradients (25ml universal) was measured. The density of band rich in Leydig cells (band 2) was determined using the standard density gradient profile. Results are presented as Mean ± SD of 3 different experiments.

Appendix 1.2. Reaction mixtures and other solutions used for 3ß-hydroxysteroid dehydrogenase (3ß-HSD), diaphorase cytochemistry, and macrophage immunocytochemistry

The methods are described in the Materials and Methods (Chapter 2). They were carried out essentially as described by Cooke *et al.*, 1983 (3ß-HSDH and diaphorase cytochemistry) and Dijkstra *et al.*, 1985 (macrophage immunocytochemistry).

A1.2.1. 3ß-HSD cytochemistry

0.1M Phosphate buffered saline (PBS) pH 7.1-7.3
One tablet of PBS (Dulbecco'A') was dissolved in 100ml water.

Reagent mixture

1) 5α-Androstene-3ß-ol -17-one (2mg) was dissolved in 1ml dimethylformamide (DMF).

2) Nitro blue tetrazolium (1mg) was dissolved in 5ml 0.1M PBS (pH 7.1-7.3) in a dark bottle and was either left at room temperature for at least 3h or at 34°C in the incubator or water bath for about 1h to dissolve. This was kept in a dark bottle.

3) Nicotinamide (1.6mg) was dissolved in 1ml PBS.

4) NAD^+ free salt (3mg) was dissolved in 1ml PBS by stirring.

The reaction mixture was made up fresh by adding to solution 2:
100µl of solution 1
700µl of solution 3
800µl of solution 4
Then this reagent (500µl) was added to 0.5×10^6 cells.

Fixative

Formaldehyde/ethanol/water (1:5:4, v/v/v)

Gelatin solution

Gelatin (15g) was dossolved in 100ml water by warming. Glycerol (100g) was added and the mixture was warmed for 5min at 60°C, then filtered through glass wool. One drop of phenol was added to 100ml of filtered solution.

Experiment	3ß-HSD positive cells	Bright ring cells
Experiment 1	17.1 ± 2.63	14.2 ± 1.02
Experiment 2	17.5 ± 3.69	14.0 ± 2.12

Table A1.2.1. Determination of Leydig cell content in unpurified interstitial cell preparations by 3ß-HSD cytochemistryand phase contrast microscopy. The Leydig cells were identified either by cytochemical staining for 3ß-hydroxysteroid dehydrogenase (3ß-HSD) or by phase contrast microscopy. The percentage of Leydig cells was determined by counting at least 5 fields of 500 cells each. Results are presented as mean ± SD of 4 aliquots taken from each experiment.

A1.2.2. Diaphorase cytochemistry (viability) Reagent mixture

Nitroblue tetrazolium (1mg) was dissolved in 6.6ml 0.1M PBS (pH 7.1-7.3) as described above (see 3ß-HSD). This was then added to a vial containing 2mg NADH disodium salt and mixed. This reagent was made up fresh just before use and 500µl were added to 10^5 cells incubated in 24-well plate.

A1.2.3. Macrophage immunocytochemistry

1) 0.01M Phosphate-buffered saline (PBS) (pH 7.4) (1l)

0.2 mol $NaH_2PO_4.2H_2O$/l	19.5ml
0.2 mol $NA_2HPO_4.12H_2O$/l	30.5ml
0.15 mol NaCl	8.76g
0.3 mmol NaN_3	20mg

2) Polysiloxone solution

Propanol/concentrated sulphuric acid/dimethyl polysiloxone (84:1:15) (v/v/v)

3) Inhibition of endogenous peroxidase

It was inhibited with either 0.1 % H_2O_2 or 0.25% periodic acid (v/v) in water.

4) Antibodies

First antibody: mouse anti-rat macrophage monoclonal antibody (ED2) was diluted 1:500 with 0.01M PBS pH 7.4.

Secondary antibody: sheep-antimouse Ig horseradish peroxidase linked $(Fab')_2$ fragment was added at a dilution of 1: 100 in 0.01M PBS pH 7.4.

5) 10mM Tris buffer pH 7.6

Tris/base (1.211g) was dissolved in 500ml water. This was adjusted to pH 7.6 with few drops of 1M HCl then made up to 1l with water.

6) DAB stock solution

Diaminobenzidine (DAB) (40mg/ml) was dissolved in ethylene glycol mono-methyl ether by stirring. This was kept at -20°C.

7) Peroxidase reagent

This was made up immediately before use: 125µl stock solution was mixed with 5µl H_2O_2 and this was made up to 5ml with 10mM Tris buffer pH 7.6.

8) Conterstaining with haematoxylin

The cells were counterstained with heamatoxylin (7.5g/l) diluted (1:3, v/v) with water for 5min. The slides were then washed in tape water and dehydrated as follows: 70% ethanol (once), 90% ethanol (twice), absolute ethanol (twice), and xylene (twice).

Cell type	Band 1	Band 2
Leydig cells (%)	12.7 ± 2.67	92 ± 4.36
Macrophages (%)	12.7 ± 3.32	3.7 ± 0.92

Table A1.2.3. Leydig cell and macrophage content in crude testicular interstitial cells purified on Percoll. Unpurified testicular interstitial cells were purified by centrifugal elutriation on Percoll gradients (0 to 90%) and band 1 (density= 1.045 g/ml) and 2 (density= 1.75 g/ml) were collected separately and aliquots from each of them were analysed for 3ß-HSD and macrophages by cytochemistry. The percentage of Leydig cells (3ß-HSD positive cells) and macrophages were determined as described in Table 3.1. The results represent pooled data from two experiments and presented as mean ± SD of cell counts from six aliquots.

Appendix 1.3. Iodination of hCG

This was carried out essentially as described by Thorell & Johansson (1971).

A.1.3.1. Buffers and solutions

1) 10µg hCG (CR127) in 50µl 0.2mol PBS/l (pH 7.4)
2) 0.5 mg/ml lactoperoxidase in 0.05 mol PBS/l
3) 0.1 % BSA in 0.01 mol PBS/l (pH 7.4)
4) 5% BSA in solution 3
5) 0.1 Ci/ml $Na^{125}I$
6) 0.2 M phosphate buffer (pH 7.5)
7) 0.007% H_2H_2 in H_2O
8) 0.1% sodium azide and 10 mmol KI/l in solution 3

A.1.3.2. Preparation of the column

PD-10 Sephadex G-25M chromatography column (PD-10) was washed with 20ml of solution 4 to block non-specific binding.

A.1.3.3. Iodination

Solution 2 (10µl), 5 (10µl), and 6 (15µl) were added to solution 1, which was in an LP4 plastic tube containing a microflea, and mixed using a magnetic stirrer. Solution 7 (10µl) was then added and the tube was covered immediately with a cap and the reaction mixture was stirred for 1min at room temperature. The reaction was stopped by addition of 1ml solution 8. Another aliquot of hCG was iodinated in the same way and the reaction was stopped by adding the solution containing the previous iodinated hCG.

A.1.3.4. Purification

The reaction mixture containing iodinated hCG from both aliquots (10µg x2) was added to the column and allowed to enter the PD-10 column. This was then eluted with solution 3 by collecting fractions of 500µl (8 drops) in the first four LP4 plastic tubes, then 250µl (4 drops) in the remaining tubes.
The radioactivity in each tube was measured using a radioisotope calibrator ^{R}CRC-10 (Capintec, ^{R}Inc, N.J., Souther Sientific LTD Worthing, Sussex) and the elution profile is shown in Figure A3a. The fractions corresponding to the peak of ^{125}I-hCG were pooled and made up to 5ml with solution 3.
The concentration of the ^{125}I-hCG was determined by measuring the binding of ^{125}I-hCG to the crude interstitial cells or elutriated/Percoll-purified Leydig cells in the presence of increasing concentrations of unlabelled pure hCG (CR-127) (0 to 5.5nmol hCG/l) as shown in Figure A3b. Then the pooled fraction of ^{125}I-hCG was diluted so that the concentration is 1ng ^{125}I-hCG/µl and stored at -20°C until used.

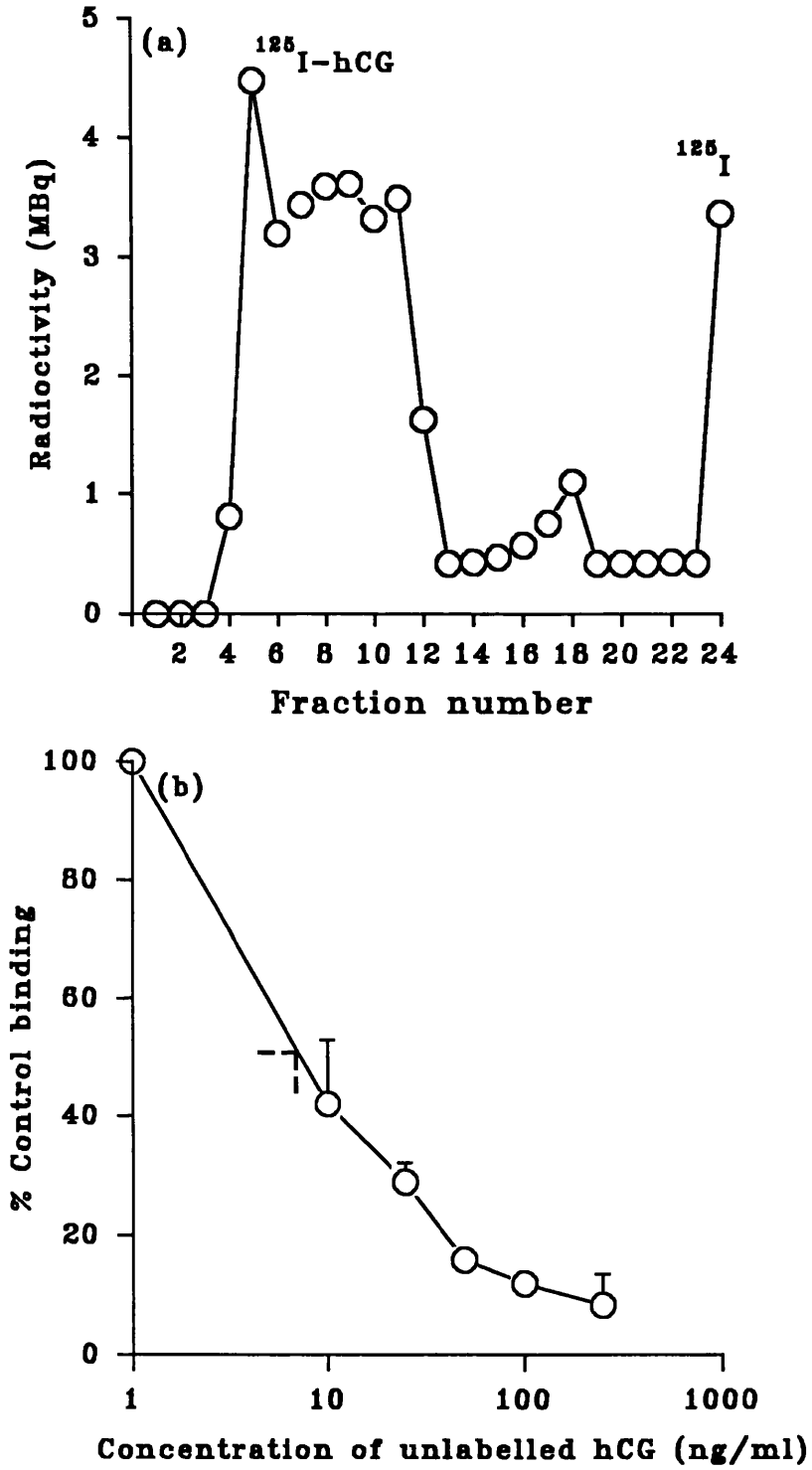


Figure A1.3. Typical elution profile of free ^{125}I and ^{125}I-labelled hCG from a PD-10 Sephadex G-25M column (a) and a competitive binding curve for the characterization of ^{125}I-labelled hCG using increasing concentrations of unlabelled hCG (CR 127) (b).

Appendix 1.4. Iodination of ScAMP-TME

This was carried out essentially as described by Brooker *et al.* (1979). For convenience (shorter time and simpler equipment for purification), another method of purification in which Sep-Pak^R cartridges (Wilson, 1988) was used instead of descending paper chromatography.

1.4.1. Buffers and solutions

1) 40µg/ml 2'-monosuccinyl adenosine-3',5'-cyclic monophosphate tyrosine methyl ester (ScAMP-TME) in 5mM sodium acetate buffer (pH 4.75)

2) 0.5 M potassium phosphate buffer pH 7.0 (100ml)
KH_2PO_4 (6.8g) was dissolved water and adjusted to pH 7.0 with KOH.M5

3) 0.1 Ci/ml $Na^{125}I$

4) 1mg/ml chromatine T in 0.5M potassium phosphate (pH 7.0)

5) 5mg/ml sodium metasulphate

6) 50mM sodium acetate buffer (pH 4.75) (100ml)
286µl acetic acid diluted with water and adjusted to pH 4.75 with NaOH.

7) 0.1M sodium acetate buffer (pH 4.75)
572µl acetic acid diluted with water and adjusted to pH 4.75 with NaOH.

8) 1-Propanol/0.1M sodium acetate solution (17.5:82.5, v/v)

A4.2. Iodination

Solution 1 (20µl), 2 (20µl), and 3 (10µl) were put into an LP4 plastic tube containing a microflea and mixed using a magnetic stirrer. Solution 4 (5µl) was then added and the tube was covered immediately with a cap and the reaction mixture was stirred for 1min at room temperature. The reaction was stopped by addition of 50µl solution 5.

A1.4.3. Purification

A1.4.3.1. Purification by descending paper chromatography

The reaction mixture was streaked onto a strip (2 x 40cm) of Watman 31 ET chromatography paper and allowed to dry under a stream of air. This was then developed by descending chromatography (20cm migration distance) using the solvent mixture butanol: glacial acetic acid: water (12:3:5) (v/v/v). Some of the solvent was used to saturate the tank used for chromatography and 100ml for the elution. After chromatography, the strip was allowed to dry partially then it was cut into 1cm segments. The latter were put separately into LP4 plastic tubes after cutting each of them into 2 pieces and counted using a CAPINTEC Radioisotope Calibrator ^{R}CRC-10 (Capintec ^{R}Inc., Southern Scientific Limited, Worthing, Sussex). The radioactive material was them eluted by addition of 1ml 50mM sodium acetate buffer (pH 4.75) to each tube. The tubes were vortexed and left at room temperature for 30min in order to elute the radioactive material. The elution profile is shown in Figure A4a. Based on previous experiments, in which the binding of the radioactive material from each fraction to the cAMP antibody was determined by RIA, the fractions in which 30 to 50% of the binding of the radioactive material to the antibody occurred (peak 2 which corresponds to the peak of ^{125}I-ScAMP-TME) were pooled, diluted with 1-propanol (1:1, v/v) and stored at -20°C until used.

A1.4.3.2. Purification using Sep-Pak^{R} C18 cartridges

After the reaction was stopped with 50µl solution 5, 800µl of solution 7 were added. The reaction mixture was applied to a C18 cartridge, previously washed with 20ml methanol and 20ml water, with a 1ml syringe. The Sep-Pak^{R} was washed first with 2.7ml solution 7 (some of the free iodine was eluted), then with 10ml of solution 8 using a 10ml syringe. One ml-fractions were collected in LP4 plastic tubes. Aliquots of 5µl from each tube were counted in a gamma counter and the elution profile is shown in Figure A4b. For storage, selected fractions were pooled, adjusted to 50% 1-propanol, and maintained at -20°C. Most of the unreacted iodine did not elute from the column.

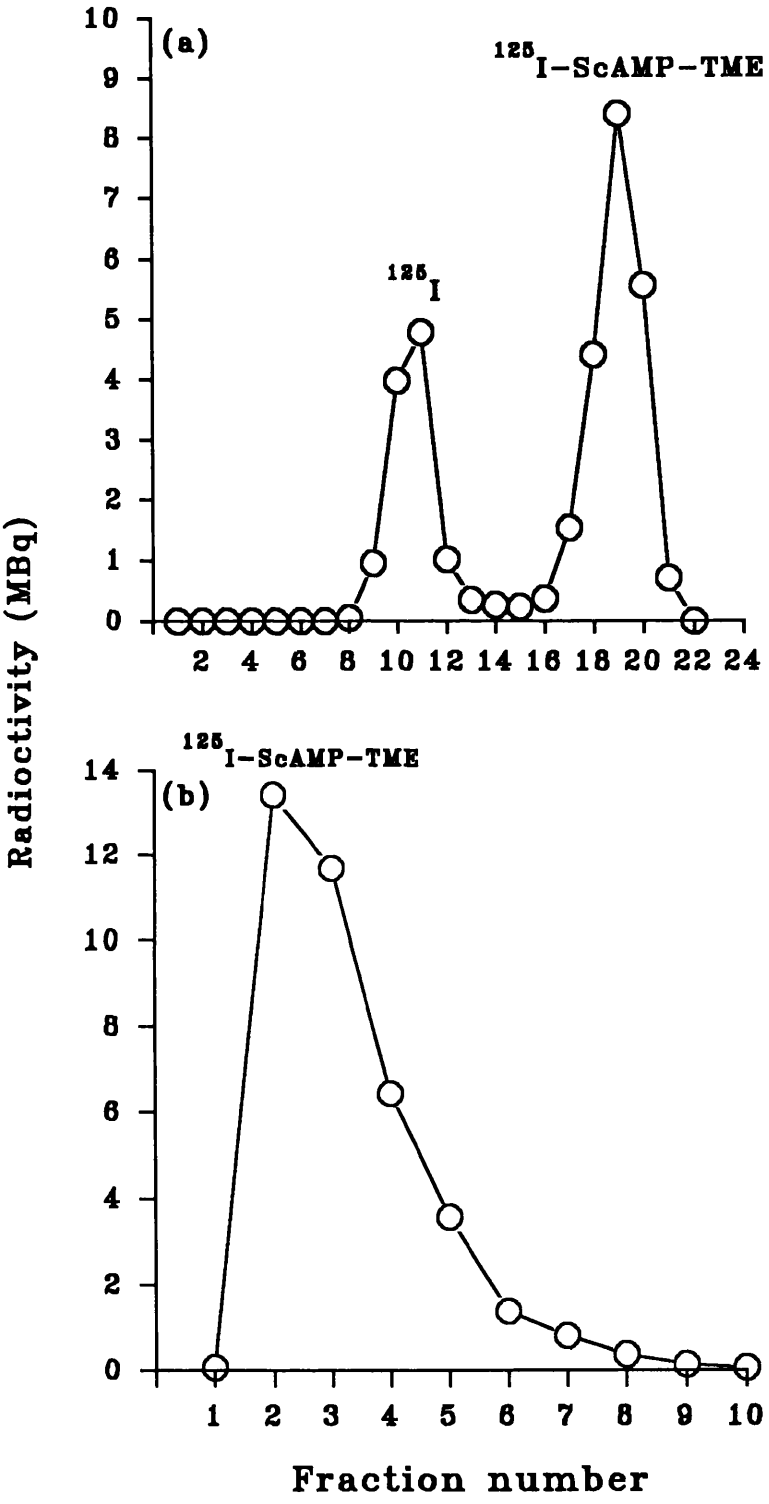


Figure A1.4. Typical profile of separation of free ^{125}I and ^{125}I-ScAMP-TME by descending paper chromatography (a) or using Sep-Pak^{R} cartridges (b).

Appendix 1.5. Cyclic AMP radioimmunoassay

This was carried out essentially as described by Steiner *et al.* (1972) and Brooker *et al.* (1979).

A1.5.1. Buffers and solutions

1) 0.1M Phosphate buffer saline (PBS) (pH 7.4) (1l)

0.2 mol $NaH_2PO_4.2H_2O$/l	195ml
0.2 mol $Na_2HPO_4.12H_2O$/l	305ml
0.15 mol NaCl	8.76g
0.3 mmol NaN_3	20mg

2) 0.3% (W/v) Bovine gamma globulin (fraction II) in PBS.

3) Sample medium
DMEM culture medium pH 7.4 containing 0.18mol $HClO_4$/l and 0.19mol K_3PO_4/l.

4) cAMP standards
Stock solutions were made up in absolute ethanol at the concentrations of 1, 10, and 100nmol/l and stored at 4°C.

5) Acetylating reagent
Acetic anhydride was mixed with triethylamine (1:2.7, v/v) immediately before use.

6) ^{125}I-ScAMP-TME
This was prepared as described above (see iodination of ScAMP-TME). The stock solution was diluted with PBS and was added in a 100µl volume containing 20,000 cpm/tube.

7) cAMP antibody (MS1)
Based on a dilution curve for each new batch of antibody, the concentration of the antibody at which the binding of about 50% of total labelled cAMP occured was selected for the RIA. The stock solution of the antibody was diluted with solution 2 to the required concentration and added in 100µl volume/tube.

8) 16% (w/v) Polyethylene glycol (PEG) in tap water.

A1.5.2. RIA procedure

Standards were aliquoted at concentrations of 10, 25, 50, 100, 250, 500, 1000, 2500, and 5000 fmol, dried under nitrogen, and redissolved in 100µl sample medium (solution 3).

Totals counts were measured by counting 100µl of ^{125}I-ScAMP-TME (20,000 cpm).

Non-specific binding (NSB) was determined by addition of 5µl acetylating reagent to 100µl sample medium. This was vortexed and left for 1h at room temperature. ^{125}I-ScAMP-TME (100µl) and 0.3% bovine gamma globulin (100µl) were then added.

Total binding (Bo) was determined by adding all the solutions added to the standards except that 100µl sample medium was added instead of unlabelled cAMP.

Samples were aliquoted out and made up to 100µl with the sample medium.

Acetylation
The acetylating reagent (solution 5) (5µl) was added to the standard or sample, mixed immediately and left at room temperature for 1h.

The antibody (100µl) and ^{125}I-ScAMP (100µl) were added to the acetylated sample or standard. All tubes were vortexed and incubated overnight at 4°C. Then Solution 8 (PEG) (1.6ml) was added to all the tubes except the totals. The tubes were vortexed and centrifuged for 30min at 3000 rpm (Sorvall^{R} RT 6000, DuPont). The supernatant was decanted and the pellet was counted for 5min in a gamma counter. The standard curve is shown in Figure 1.5.

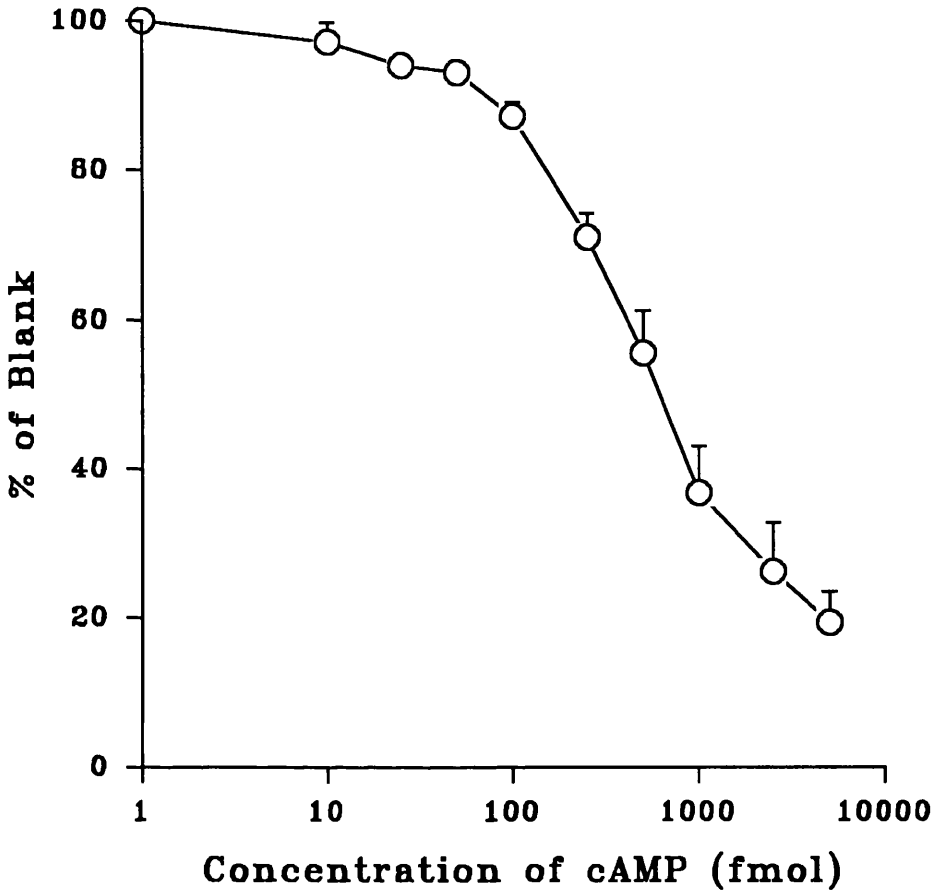


Figure A1.5. Typical standard curve for the radioimmunoassay of cAMP. Results are presented as means ± SD of 3 different experiments. Total counts, non-specific binding (NSB), and blank were 22824 ± 3619, 713 ± 245, and 10997 ± 1775 cpm respectively.

Appendix 1.6. Pregnenolone and testosterone radioimmunoassay

A1.6.1. Buffers and solutions

1) 0.01M Phosphate buffer (pH 7.0) (1l)
0.2mol $NaH_2P0_4.2H_2O$/l (19.5ml)
0.2mol $Na_2HP0_4.12H_2O$/l (30.5ml)

2) Pas-gel
0.01 M phosphate buffer (pH 7.4) (1l)
0.14mol NaCl/l (8.19g)
0.3mmol NaN_3 (20mg)
0.1% gelatin (1g) was added after being dissolved in a small volume of phosphate buffer by warming

3) Standard pregnenolone and testosterone stock solutions were made up in absolute ethanol at a concentration of 5ng/ml and stored at 4°C.

4) ^{3}H-Pregnenolone and ^{3}H-testosterone
The stock solution was diluted with Pas-gel and added in a volume of 100µl containing 15,000 dpm.

5) Pregnenolone and testosterone antibodies
Based on a dilution curve for each new batch of antibody, the dilution of the antibody at which the binding of about 50% of the total ^{3}H-pregnenolone or ^{3}H-testosterone occured, was selected for theRIA. The stock solution of the antibody was diluted with Pas-gel to the required concentration and added in a 100µl volume/tube.

6) Sample medium same as described above (see cAMP RIA)

7) Charcoal solution
5mg dextran T500 was dissolved in 20ml 0.01M phosphate buffer pH 7.0 by stirring. Activated charcoal (50mg) was then added, mixed and added in a 500µl volume/tube.

A1.6.2. RIA procedure

Standards were aliquoted at concentrations of 100, 200, 300, 400, 500, 600, and 800 pg (pregnenolone) and 25, 50, 75, 100, 125, 150, 175, 200 and 250pg (testosterone), dried under nitrogen, and redissolved in 100µl sample medium.

Controls

Totals
100µl sample medium
100µl Pas-gel
100µl ^{3}H-pregnenolone or ^{3}H-testosterone

NSB
100µl sample medium
100µl Pas-gel
100µl ^{3}H-pregnenolone or ^{3}H-testosterone

Total binding (Bo) was determined by adding all the solutions added to the standards except that 100µl sample medium was added instead of unlabelled pregnenolone or testosterone.

Samples were aliquoted out and made up to 100µl with the sample medium.

The antibody (100µl) for pregnenolone or testosterone and 100µ of ^{3}H-pregnenolone or ^{3}H-testosterone were added to the sample or standard. All tubes were vortexed and incubated overnight at 4°C. Then the charcoal solution (500µl/tube) was added to all except the totals. The tubes were vortexed and centrifuged for 10min at 3000 rpm (Sorvall^{R} RT 6000, DuPont). The supernatant was decanted into a mini scintillation vials. Scintillation fluid (Ultma Gold) (2ml/vial) was added and the vials were vortexed then counted for 5min in a Beckman ß-scientillation counter.
The standard curve for pregnenolone (a) and testosterone (b) are shown in Figure 1.6.

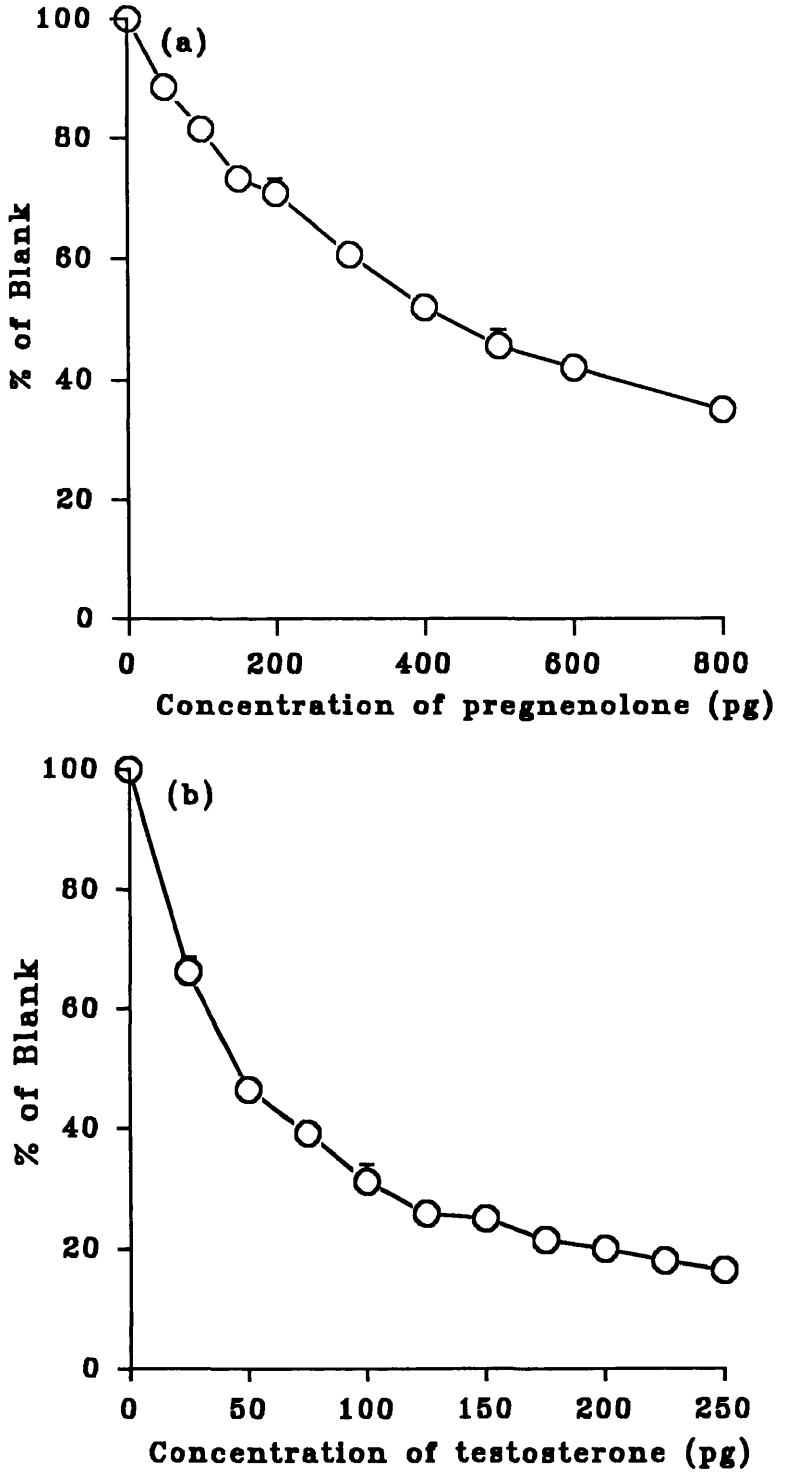


Figure A1.6. Typical standard curve for the RIA of pregnenolone (a) and testosterone (b). Results are presented as means ± SD of 3 different experiments. Total counts, NSB, and blank were 16099 ± 1001, 134 ± 13.3, and 10803 ± 155 dpm for pregnenolone and 21220 ± 963, 310 ± 35.5, and 9462 ± 231 dpm for testosterone respectively.

Appendix 1.7. Aromatase activity

The assay was described in Materials and Methods (Chapter 2).

	Recovery of ^3H_2O (dpm)
Total ^3H_2O	1570777 ± 63826
^3H_2O recovered after incubation for 2h	1656733 ± 60723
^3H_2O	1574399 ± 74254

Table A1.7. Determination of a possible loss in ^3H_2O resulting from aromatization by evaporation during incubation at 34°C for 2h. ^3H_2O (1μCi) was added to 4 x 10^5 Leydig cells incubated in 1ml DMEM in 24 well plates at the start or at the end of the incubation period. The cells were then subjected to the same procedure as described in Materials and Methods (Chapter 2). Results are presented as means ± SD of triplicate incubations.

Appendix 1.8. Protein synthesis (amino acid incorporation)

A1.8.1. Culture media and other solutions

1) DMEM/F12 culture medium (pH 7.4) (1l) (methionine-free medium)
14.8g of DMEM/F12 base mixture with 15mM Hepes was substituted with:
350mg L-glutamine
50mg L-leucine
240 L-lysine
90.61 $CaCl_2$
112.6mg $MgCl_2$
97.67mg $MgSO_4$
1g BSA.

2) DMEM/F12/methionine culture medium (pH 7.4) (1l)
1.49g L-methionine (10mmol/l) was added to medium 1

3) PBS/BSA/methionine solution (pH 7.4) (500ml)
7.06g Na_2HPO_4 $7H^{20}$
4.5g NaCl
0.5g BSA
746 mg L-methionine

4) 10% TCA in water (w/v)/ methionine solution (500ml)
50g TCA
746mg L-methionine

A1.8.2. The assay is described in Materials and Methods (chapter 2)

A1.8.3. Bio-Rad protein assay

The dye reagent concentrate was diluted 1:5 in water and filtered. Aliquots of the cell homogenates were diluted 1:10 with water. The protein content was determined using a standard curve for BSA (fraction V) in a range of 0-1µg/ml. Standard or sample was added in a 20µl volume to 480µl diluted dye, mixed, and incubated for 20min. The absorbance was measured at 595nm.

Appendix 1.9. Histological procedure

A1.9.1. Solutions

Bouins Solution (fixative)
Saturated watery picric acid/ formaldehyde/ acetic acid (75:25:5, v/v/v). This was prepared fresh just before use.

Schiff's reagent
Solution 1: 0.5g pararosaniline (CI 42500) in 15ml HCl.
Solution 2: 0.5g potassium metasulphite in 85ml water
Solution 1 was added to solution 2 and kept in the dark for 24h. This was then decolourised with 200mg charcoal for up to 2min and filtered.

Scott's water
2g $NaCO_3$ and 10g $MgSO_4$ $7H_2O$ were dissolved in 1l tap water.

A1.9.2. Procedure

The testes were fixed in Bouins solution for 48h then kept in 70% ethanol (ETOH) untill processed. Each testis was cut into two parts and a 5-10mm thick slices was further processed. The tissue from each testis was put in a 25ml glass vial and dehydrated as follows:

a) 20ml water, once for about 2h
b) 15ml 1% lithium carbonate, once for 2 to 4h or overnight.
c) 20ml water, 3 to 4 times for 15min each until the picric acid was almost removed.
d) 70% ETOH, 3 times for 20min each
e) 90% ETOH, 3 times for 20min each
f) 100% ETOH, 3 times for 20min each

At all stages of dehydration, the samples were mixed using a rotating mixer. The dehydrated material was preembedded in Technovit 7100 plastic, a glycol methacrylate (10ml per sample) to which harder component I was added. This was left overnight at room temperature mixing using a rotating mixer. The tissue was then removed and put into chambres where it was embedded using a mixture of Technovit 7100 and a harder component II (15:1, v/v). This was left for 2 to 4h at room temperature until the polymerization was nearly completed. Then this was left in the incubator at 37°C overnight or longer. A plastic self mounting sample holder was put on the top of each sample chambre. This was filled with a resine mixture Technovit 3040 and was left for 15min to solidify. Five µm sections were cut with an 8mm glass thich knife, at least 1mm away from the edge where the testis was cut in half, in order to avoid damage to the interstitial compartment. The sections were first put in water to spread, then transferred to slides and dried on a hot plate. The slides were then put in racks and kept in an incubator at 37°C overnight.

A1.9.3. Staining using the periodic acid-Shiff's (PAS) technique and Gill's haematoxylin

The staining was carried out as follows:

a) The slides were washed with water, then stained with a preheated 1% periodic acid for 30min at 50°C mixing every 5 to 10min. This was then washed with running tap water for 5min followed by distilled water.

b) The tissue sections were stained with Shiff's reagent for 30 to 40min at room temperature mixing every 5 to 10min. The slides were washed with running water for 15min followed by distilled water.

c) The tissue sections were stained with Gill's haematoxylin (number 3) for 10min at room temperature mixing every 2min. This was washed 3 to 4 times with distilled water, once with Scott's tap water for 5min, and then with distilled water.

d) The slides were dried out on a hot plate mounted in manitol and put back on the hot plate before they were covered with coverslips.

APPENDIX 2: CHAPTER 4 and 5

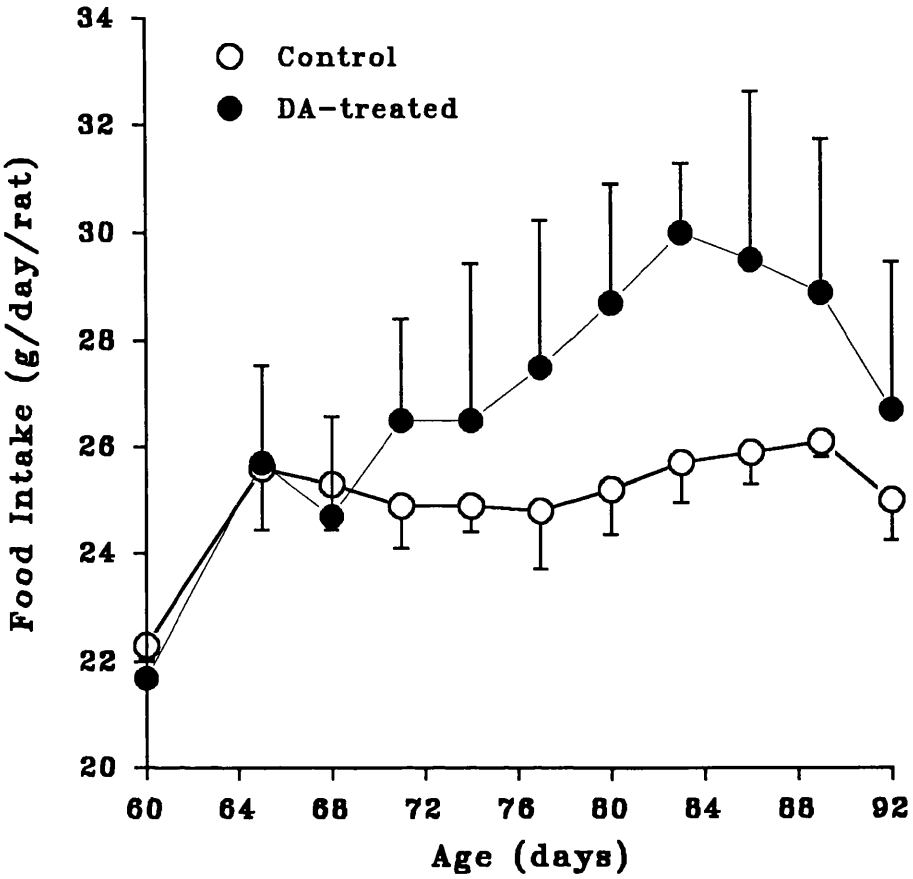
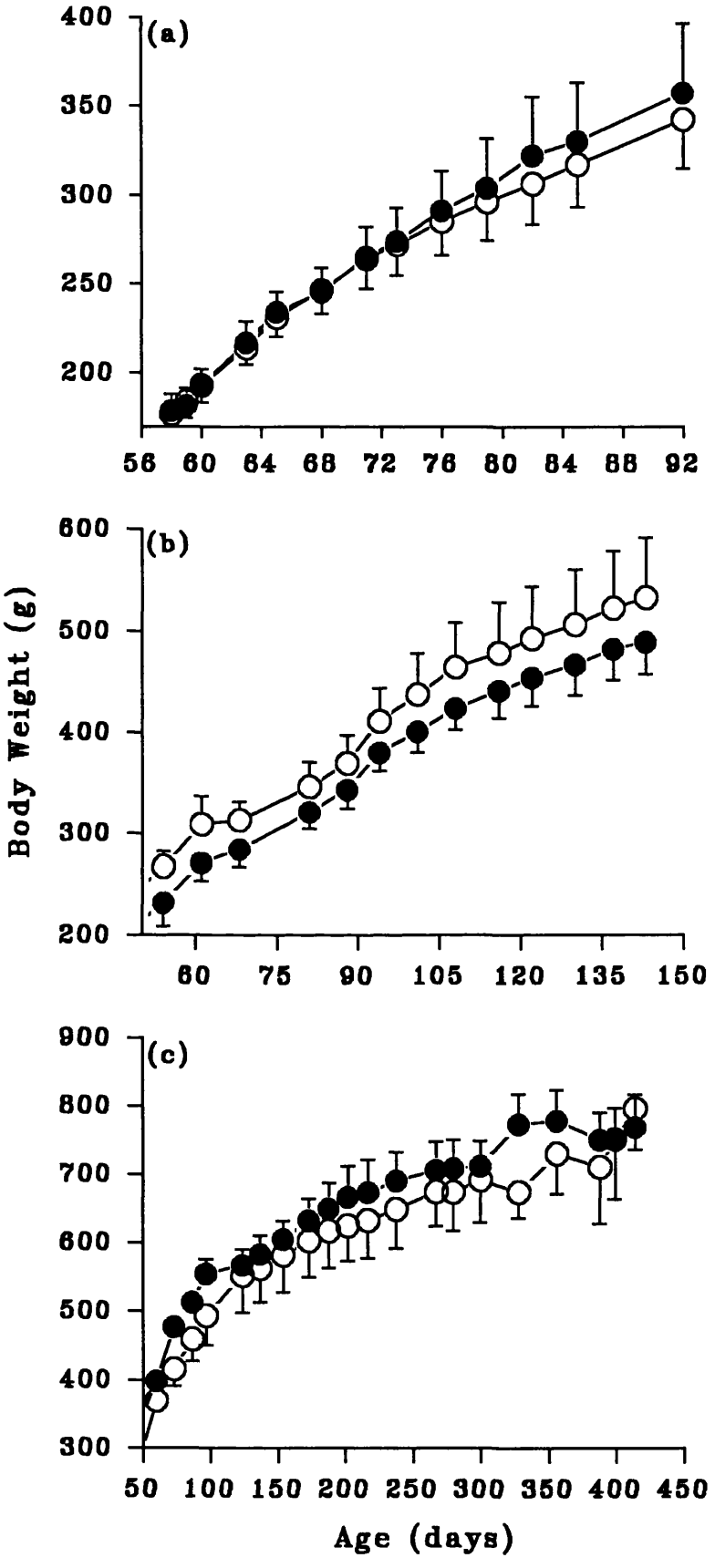


Figure A2.1. Food intake in controls and rats treated with a dopamine agonist (DA) (CU 32-085).

Each point represents the mean (± SD) food intake per rat of 3 groups from controls and DA-treated. The treated animals were given the DA (2mg/kg body weight/day) for 4 weeks starting at 65 days of age.


(a)
400
350
300
250
200
56 60 64 68 72 76 80 84 88 92
(b)
600
500
400
300
200
60 75 90 105 120 135 150
Body Weight (g)
(c)
900
800
700
600
500
400
300
50 100 150 200 250 300 350 400 450
Age (days)

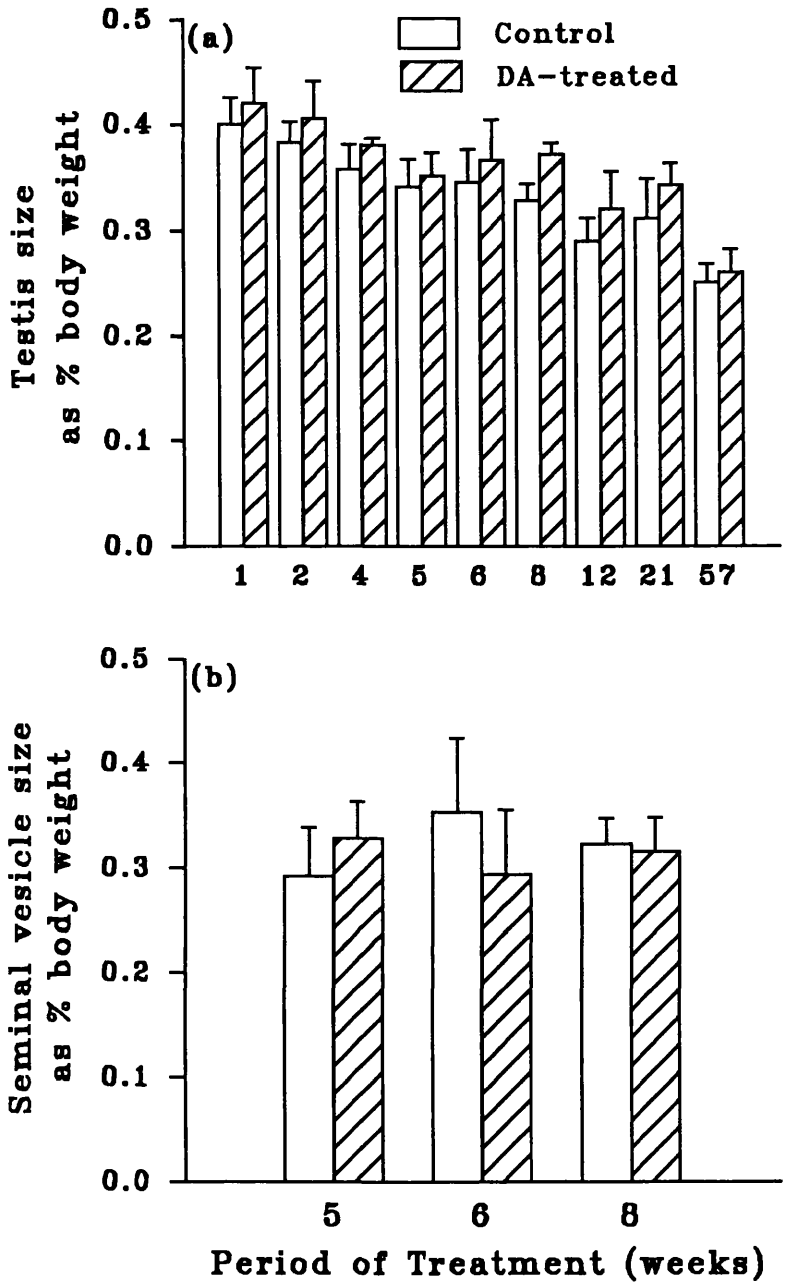


Figure A2.3. Testicular (a) and seminal vesicle (b) size in control and DA-treated rats.

The results are presented as mean (± SD) testicular or seminal vesicle size (expressed as % body weight) of at least three different experiments. Five to ten rats per group were used in each experiment and both testes and seminal vesicles were weighed at the end of the period of treatment.

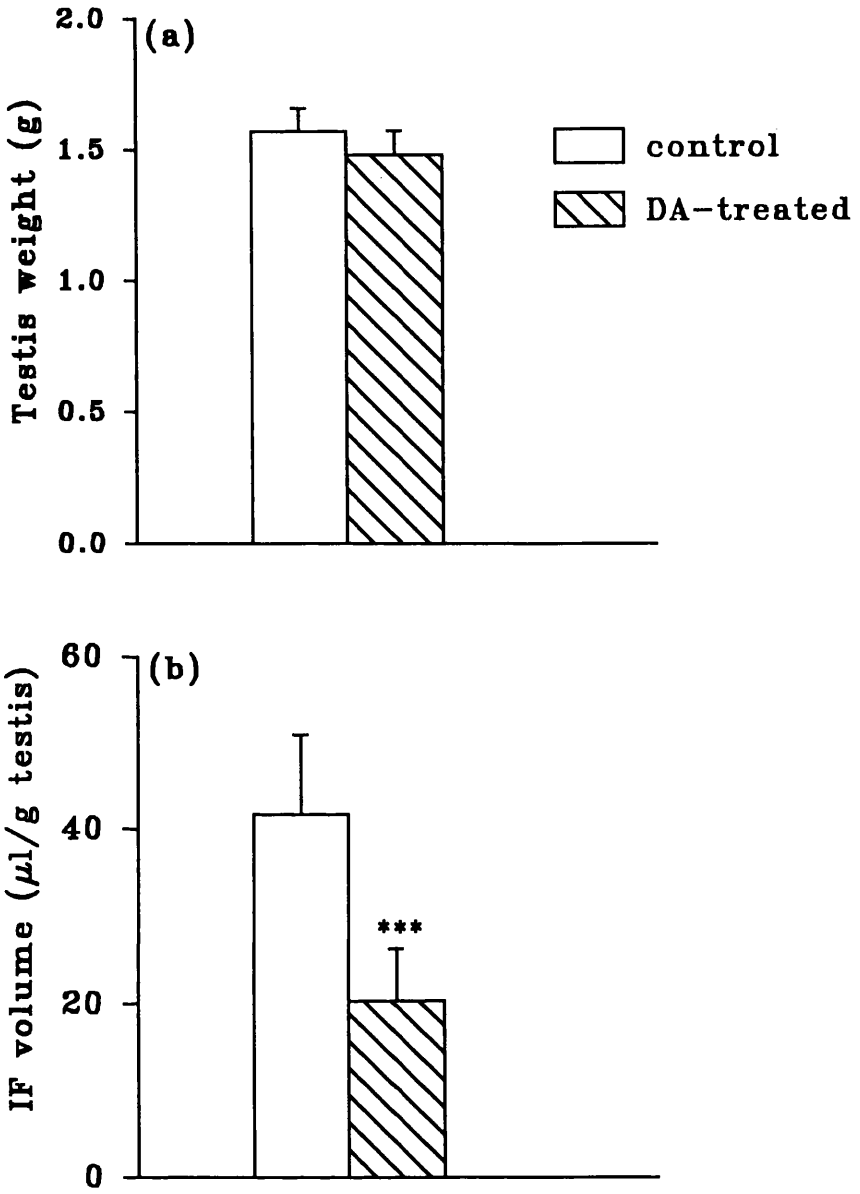


Figure A2.4. Testicular weight (a) and interstitial fluid volume (b) in control rats and rats treated with the DA for 5 weeks.
The interstitial fluid was measured as described in Materials and Methods (Chapter 2). Values represent means ± SD, n=5 rats per group. *** P<0.001 compared with the control group.

Experiment	Viability	
	Control	DA-treated
Experiment 1	97.9 ± 0.67	98.1 ± 0.74
Experiment 2	98.8 ± 0.55	98.5 ± 0.96
Experiment 3	97.7 ± 1.78	95.5 ± 1.78

Table A2.1. Viability of elutriated/Percoll-purified Leydig cells, from testes of control rats and rats treated with the DA for 5 weeks. The percentage of viable cells was determined by diaphorase cytochemistry. Aliquots from the elutriated/Percoll-purified fraction corresponding to fractions F4 to F8 were analysed for the viability. The percentage of viable cells was determined by counting at least 5 fields of 500 cells each. Results are presented as means ± SD of 3 different cell aliquots assayed in duplicate in each experiment.

Group	Total number of animals	Number of animals dead	Percentage of death (%)
Control	10	8	80
DA-treated	10	2	20

Table A2.2. Incidence of death in control rats and rats treated with the dopamine agonist for 71 weeks.

APPENDIX 3: CHAPTER 6

Group	Body weight at the start of treatment (g)	Body weight at the end of treatment (g)	Weight gained or lost (g)
Control	478 ± 24.9	501 ± 20.8	+22.4 ± 5.46
hCG-treated	407 ± 13.5	417 ± 16.9	*** +9.6 ± 7.50
(hCG/Dex)-treated	468 ± 23.4	379 ± 22.2	*** -89.8 ± 2.58
Dex-treated	491 ± 37.9	395 ± 26.9	*** -96.2 ± 11.38

Table A3.1. Body weight in control rats and rats treated with hCG and/ or dexamethasone for one week. The rats were weighed, then were given the vehicle (control), 100IU hCG with or without dexamethasone (Dex) (1mg/kg body weight), or Dex alone once per day for one week. The animals were weighed at the end of treatment and the change in body weight in response to treatment with hCG and or Dex was determined. Values represent means ± SD body weight of 5 rats from each group. ***$p<0.001$ compared with the control group.

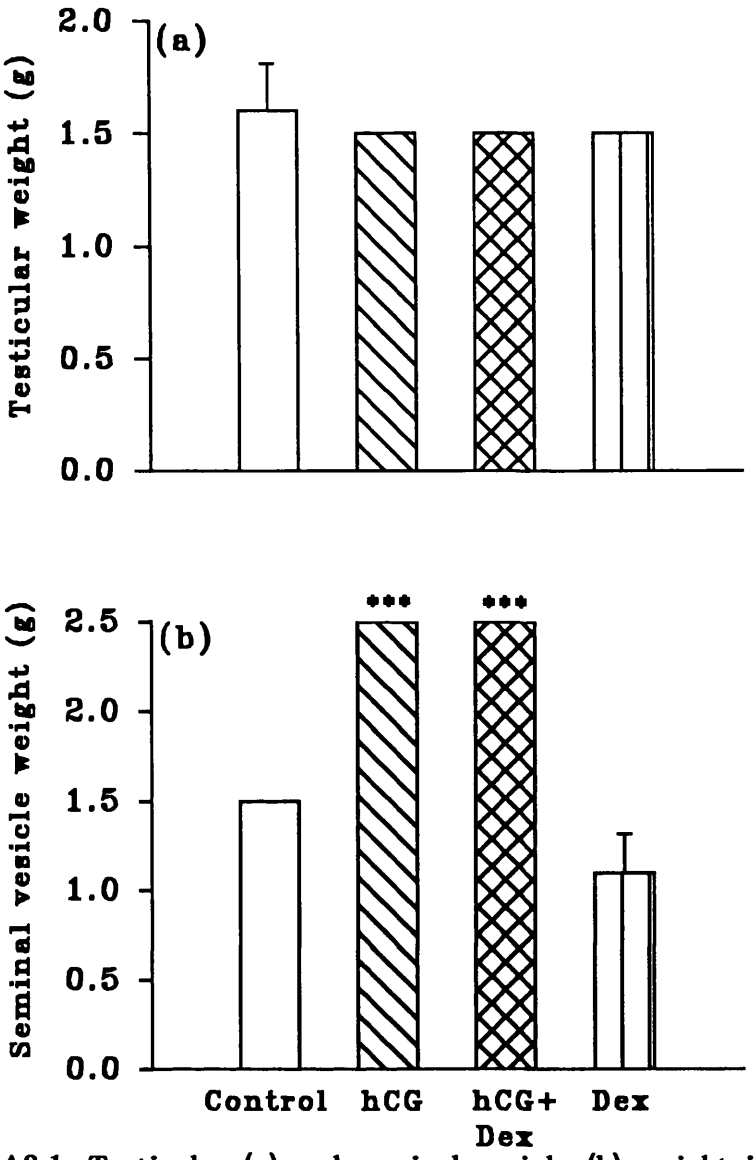


Figure A3.1. Testicular (a) and seminal vesicle (b) weight in control rats and rats treated with hCG and/or dexamethasone (Dex). The rats were given hCG (100 IU), dexamethasone (1mg/kg body weight), and hCG + Dex once per day for 1 week. Five rats per group were used in each experiment and both testes and seminal vesicles were weighed at the end of the period of treatment. Results are presented as mean (±SD) of testicular or seminal vesicle weight of pooled data from two experiments. ***P<0.001 compared with the controls and the group treated with dexamethasone.

Group	Period of treatment	
	6h % Viable Cells	1 week % Viable Cells
Control	91.9	95.8
hCG-treated	92.9	96.4
(hCG + Dex)-treated	92.2	97.9
Dex-treated	94.2	97.4

Table A3.2. Viability of elutriated/Percoll-purified Leydig cells from testes of control rats and rats treated with hCG and/or dexamethasone. The percentage of viable cells was determined by diaphorase cytochemistry. Aliquots from the elutriated/Percoll-purified fractions were analysed for the viability. The percentage of viable cells was determined by counting at least 5 fields of 500 cells each. Results are presented as means of pooled data from two different experiments each performed in triplicate.

Group	Crude testicular interstitial cells		Purified Leydig cells
	Percentage (%) of macrophages	Number of macrophages per testis ($x10^{-6}$)	Percentage of macrophage (%)
Control	7.4	5.5	6.0
hCG-treated	3.9	4.9	4.1
(hCG + Dex)-treated	6.2	3.7	7.5
Dex-treated	7.9	5.1	6.1

Table A3.3. Macrophage content in crude testicular interstitial cells and in elutriated/Percoll-purified Leydig cell preparations. The macrophages were characterized by immunocytochemistry using a macrophage monoclonal antibody ED2. The number and percentage of macrophages were determined by counting at least 5 fields of 500 cells each. Results are presented as means of pooled data from two different experiments, each performed in triplicate.

Abayasekara, D.R.E., Band, A.M. & Cooke, B.A. (1990). *Mol. Cell. Endocrinol.* ***70, 147.***

Abayasekara, D.R.E., Kurlak, L.O., Jeremy, J.Y., Dandona, P., Sharpe, R.M. & Cooke, B.A. (1990). *Int. J. Androl.* ***13, 408.***

Abbot, S.D., Docherty, K. & Clayton, R.N. (1988). *J. Mol. Endocrinol.* ***1, 61.***

Abney, T.O. & Melner, M.H. (1979). *Steroids* ***24, 413.***

Abney, T.O. & Carswell, L.S. (1986). *Mol. Cell. Endocrinol.* ***45, 157.***

Ackerman, G.E., Smith, M.E., Mendelson, C.R., MacDonald, P.C. & Simpson, E.R. (1981). *J. Clin. Endocrinol. Metab.* ***53, 412.***

Ackland, J.F. & Schwartz, N.B. (1991). *Biol. Reprod.* ***45, 295.***

Adams, E.F., Coldham, N.G. & James, V.H.T. (1988). *J. Endocrinology* ***118, 149.***

Adashi, E.Y. & Hsueh, A.J.W. (1981). *Endocrinology* ***109, 1793.***

Adashi, E.Y. & Hsueh, A.J.W. (1981). *Nature* ***293, 650.***

Adashi, E.Y. & Hseuh, J.W. (1981). *Nature* ***293, 737.***

Adashi, E.Y., Fabics, C. & Hsueh, A.J.W. (1982). *Biol. Reprod.* ***26, 270.***

Adashi, E.Y. & Hsuch, A.J.W. (1982). *J. Biol. Chem.* ***257, 1301.***

Adashi, E.Y. & Resnick, C.E. (1986). *Endocrinology* ***119, 1879.***

Adashi, E.Y., Resnick, C.E., Croft, C.S. & Payne, D.W. (1989). *J. Biol. Chem.* ***264, 11591.***

Adashi, E.Y., Resnick, C.E., Hernandez, E.R., May, J.V., Purchio, A.F. & Twardzik, D.R. (1989). *Mol. Cell. Endocrinol.* ***61, 247.***

Adashi, E.Y., Resnick, C.E., Cronin, M.J. & Hewlett, E.L. (1984). *Endocrinology* ***115, 839.***

Adinolfi, M. (1988). *Develop. Med. Child Neurol.* ***30, 536.***

Ailenberg, M., McCabe, D. & Fritz, I.B. (1990). *Endocrinology* ***126, 1561.***

Ailenberg, M., Tung, P.S., Pelletier, M. & Fritz, I.B. (1988). *Endocrinology* ***122, 2604.***

Akhtar, M., Calder, M.R., Carina, D.L., Wright, J.N. (1982). *Biochem. J.* ***201, 569.***

Albert, D.H., Ascoli, M., Puett, D. & Coniglio, J.G. (1980). *J. Lipid Res.* ***21, 862.***

Aldred, L. F. & Cooke, B. A. (1982). *Int. J. Androl.* ***5, 191.***

Aldred, L.F. & Cooke, B.A. (1983). *J. Steroid Biochem.* ***18, 411.***

Allenby, G., Foster, P.M.D. & Sharpe, R.M. (1991). *Endocrinology* ***128, 467.***

Amador, A., Steger, R.W., Bartke, A., Johns, A., Siler-Khodr, T.M., Parker, C.R. & Shepherd, A.M. (1985). *J. Androl.* ***6, 61.***

Ambrosi, B., Giovine, C., Nava, C., Elli, R. & Vaglia, G. (1985). *Acta Endocrinol.* ***109, 1.***

Amsterdam, A. & Suh, B.S. (1991). *Endocrinology* ***128, 503.***

Anakwe, O.O. & Payne, A.H. (1987). *Mol. Endocrinol.* ***1, 595.***

Andersen, P.H., Gingrich, J.A., Bates, M.D., Dearry, A., Falardeau, P., Senogles, S.E. & Caron, M.G. (1990). *TIPS* ***11, 231.***

Anderson, C.M. & Mendelson, C.R. (1985). *Arch. Biochem. Biophy.* ***238, 378.***

Andreis, P.G., Cavallini, L., Malendowicz, L.K., Bebuffat, P., Mazzocchi, G. & Nussdorfer, G.G. (1989). *J. Submicrosc. Cytol. Pathol.* ***21, 703.***

Aoki, A. & Massa, E.M. (1972). *Arch. Histol. Jpn.* ***42, 51.***

Aoki, A. & Fawcett, D.W. (1978). *Biol. Reprod.* ***19, 144.***

Aquilano, D.R. & Dufau, M.L. (1983). *Endocrinology* ***113, 94.***

Aquilano, D. R. & Dufau, M. L. (1984). *Endocrinology* ***114, 499.***

Aquilano, D.R., Tsai-Morris, C.-H., Hattori, M.-A. & Dufau, M.L. (1985). *Endocrinology* ***116, 1745.***

Aragona, C. & Friesen, H.G. (1975). *Endocrinology **97**, 677.*

Arimura, A., Serafini, P., Talbot, S. & Schally, A.V. (1979). *Biochim. Biophys. Res. Commun. **90**, 687.*

Arthur, J.R. & Boyd, G.S. (1974). *Eur. J. Biochem. **49**, 117.*

Ascoli, M. (1981). *J. Biol. Chem. **256**, 179.*

Ascoli, M., Euffa, J. & Segaloff, D.L. (1987). *J. Biol. Chem. **262**, 9196.*

Ascoli, M., Pignataro, O.P. & Segaloff, D.L. (1989). *J. Biol. Chem. **264**, 6674.*

Ascoli, M. & Puett, D. (1978). J. Biol. Chem **253**, 4892.

Ascoli, M. & Segaloff, D.L. (1986). *J. Biol. Chem. **261**, 3807.*

Ascoli, M. & Segaloff, D.L. (1989). *Endocr. Rev. **10**, 27.*

Audi, L., Toràn, N., Vicens, E., Martinez-Mora, J., Reventos, J., Potau, N., Carrascosa, A., Albisu, M. & Gusiné, M. (1984). *In: Recent Progress in Cellular Endocrinology of the Testis, Vol **123**, pp 483-490. INSERM.*

Avallet, O., Vigier, M., Perrard-Sapori, M.H. & Saez, J.M. (1987). *Biochim. Biophy. Res. Comm. **146**, 575.*

Avallet, O., Perrar-Sapori, M.H., Vigier, M., Skalli, M. & Saez, J.M. (1988). *In: Molecular and cellular endocrinology of the testis. Vol 50, pp 319-324. Eds B.A. Cooke & R.M. Sharpe. Serono Symposia: Raven Press.*

Axelrod, J., Burch, R.M. & Jelsema, C.L. (1988). *Trends Neurol. Sci. **11**, 117.*

Ayer-Lelievre, C., Olson, L., Ebendal, T., Hallböök, F. & Persson, H. (1988). Proc. Natl. Acad. Sci. USA **85**, 2628.

Azad, N., Emanuele, N.V., Halloran, M.M., Tentler, J. & Keller, M.R. (1991). *Endocrinology **128**, 1679.*

Baba, S., Paul, H.J., Pollow, K., Jaretschek, G., Jacobi, G.H. (1981). *Prostate 2, 163.*

Bagavandoss, P., Wiggins, R.C., Kunkel, S.L., Remick, D.G. & Keyes, P.L. (1990). *Biol. Reprod. **42**, 367.*

Baird, D.R., Galbraith, A., Fraser, I.S. & Newsam, J.E. (1973). *J. Endocrinol.* ***57, 285.***

Baird, J.G., Dobson, P.R.M. & Brown, B.L. (1985). *J. Endocrinol.* ***104 (Supll.),*** *Abstract 107.*

Bakker, C.P., van der Plank-van Winsen, M.P.I. & van der Molen, H.J. (1979). *Biochim. Biophs. Acta* ***584, 94.***

Baldessarini, R.J. (1985). *In: The Pharmacological basis of therapeutics (Gilman, A.G. et al., eds) pp 387-445. MacMillan: New York.*

Baldwin, D.M. (1979). *Endocrinology* ***105, 120.***

Baldwin, D.M. & Sawyer, C.H. (1974). *Endocrinology* ***94, 1397.***

Bambino, T.H., Schreiber, J.R. & Hsueh, A.J.W. (1980). *Endocrinology* ***107, 908.***

Bambino, T.H. & Hsueh, A.J.W. (1981). *Endocrinology* ***108, 2142.***

Ban, E., Crumeyrolle-Arias, M., Latouche, J., Leblanc, P., Heurtier, J.F., Drieu, Fillion, G. & Haour, F. (1990). *Mol. Cell. Endocrinol.* ***70, 99.***

Barb, C.R., Kraeling, R.R., Rampacek, G.B., Fonds, E.S. & Kiser, T.E. (1982). *J. Reprod. Fert.* ***64, 85.***

Barkey, R.J., Shani, J., Amit, T., Barzilai, D. (1977). *J. Endocrinol.* ***74, 163.***

Barkey, R.J., Shani, J. & Barzilai, D. (1979). *J. Endocrinol.* ***81, 11.***

Barkey, R.J., Weiss-Messer, E., Mandel, S., Gahnem, F. & Amit, T. (1987). *Mol. Cell. Endocrinol.* ***52, 71.***

Barnes, R.B., Artal, R. & Lobo, R.A. (1987). *Obst. & Gynecol.* ***70, 153.***

Barnes, R.B., Cha, K.-Y., Lee, D.G. & Lobo, R.A. (1986). *Am. J. Obst. Gynecol.* ***154, 445.***

Baron, J., Taylor, W.E. & Masters, B.S.S. (1972). *Arch. Biochem. Biophy.* ***150,*** *105.*

Barraclough, C.A., Wise, P.M. & Selmanoff, M.K. (1984). *Rec. Prog. Horm. Res.* ***40, 487.***

Bartke, A. (1971). *J. Reprod. Fert.* ***25, 153.***

Bartke, A., Sweeney, C.A., Johnson, L., Castracane, V.D. & Doherty, P.C. (1985). *Exp. Aging Res.* ***11, 123.***

Beck, W. & Wuttke, W. (1977). *J. Endocrinol.* ***74, 67.***

Beck, C.A. & Garner, C.W. (1989). *Mol. Cell. Endocrinol.* ***63, 93.***

Bellvé, A.R. & Zheng, W. (1989). *J. Reprod. Fert.* ***85, 771.***

Belhadj, H., de Besi, L., Bardin, C.W. & Thau, R.B. (1989). *J. Endocrinol.* ***121,*** *451.*

Bélisle, S., Bellabarba, D., Lehoux, J.-G. & Gallo-Payet, N. (1991). *Mol. Cell. Endocrinol.* ***76, 63.***

Bellvé, A.R. & Feig, L.A. (1984). *Rec. Prog. Horm. Res.* ***40, 531.***

Belz, G., Tsai, P. & Weakley, R. (1976). *J. Biol. Chem.* ***251, 2839.***

Benahmed, M., Bernier, M., Ducharme, J.R. & Saez, J.M. (1982). *Mol. Cell. Endocrinol.* ***28, 705.***

Benahmed, M., Morera, A.M., Chauvin, M.C. & de Peretti, E. (1987). *Mol. Cell. Endocrinol.* ***50, 69.***

Benahmed, M., Reventos, J. & Saez, J.M. (1983). *Endocrinology* ***112, 1952.***

Benahmed, M., Reventos, J., Tabone, E. & Saez, J.M. (1985). *Am. J. Physiol.* ***248, E176.***

Ben-Jonathan, N. (1985). *Endocr. Rev.* ***6, 564.***

Bercovici, J.P., Tater, D., Khoury, S., Charles, J.F., Floch, J., Leroy, J.P. (1981). *J. Clin. Endocrinol. Metab.* ***53, 1291.***

Bergh, A. (1982). *Int. J. Androl.* ***5, 325.***

Bergh, A. (1983). *Int. J. Androl.* ***6, 57.***

Bergh, A. (1985). *Int. J. Androl.* ***8, 86.***

Bergh, A. (1987). *Int. J. Androl.* ***10, 765.***

Berkovitz, G.D., Fujimoto, M., Brown, T.R., Brodie, A.M. & Migeon, C.J. (1984). *J. Clin. Endocrinol. Metabol.* ***59, 665.***

Berkovitz, G.D., Carter, K.M., Brown, T.R. & Migeon, C.J. (1990). *Mol. Cell. Endocrinol.* ***69, 187.***

Berridge, M.J. (1984). *Biochem. J.* ***220, 345.***

Berridge, M.J. (1986). *Cancer Surveys* ***55, 413.***

Berridge, M.J. (1987). *Ann. Rev. Biochem.* ***56, 159.***

Besman, M.J., Yanagibashi, K., Lee, T.D., Kawamura, M., Hall, P.F. & Shively, J.E. (1989). *Proc. Natl. Acad. Sci. USA* ***86, 4897.***

Bevilacqua, M., Vago, T., Scorza, D. & Norbiato, G. (1982). *Biochim. Biophys. Res. Commun.* ***108, 1661.***

Bhalla, V. K., Flasch, M. V., Browne, E. S., Sohal, G. S., Sharawy, M. M. (1987). *J. Biol. Chem.* ***262, 5322.***

Bicsak, T.A., Tucker, E.M., Cappel, S., Vaughan, J., Rivier, J., Vale, W. & Hsueh, A.J.W. (1986). *Endocrinology* ***119, 2711.***

Birnbaumer, L. (1990). *FASEB J.* ***4, 3068.***

Birnbaumer, L., Codina, J., Mattera, R., Cerione, R., Hildebrandt, J., Sunyer, T., Rojas, F.J., Caron, M.G., Lefkowitz, R.J. & Iyengar, R. (1985). *Rec. Prog. Horm. Res.* ***41, 41.***

Bishop J.M. (1987). *Science* ***235, 305-311.***

Bjorklund, A., Moore, R.Y., Nobin, A. & Stenevi, V. (1973). *Brain Res.* ***51, 171.***

Blank, M.S., Fabbri, A., Catt, K.J., Dufau, M.L. (1986). *Endocrinology* ***118, 1097.***

Blok, L.J., Mackenbach, P., Trapman, J., Themmen, A.P.N., Brinkmann, A.O. & Grootegoed, J.A. (1989). *Mol. Cell. Endocrinol.* ***63, 267.***

Blonquist, C.H., Linderman, N.J. & Hakanson, E.Y. (1987). *J. Clin. Endocrinol. Metab.* ***65, 647.***

Bogovich, K. & Payne, A.H. (1980). *J. Biol. Chem.* ***255, 5552.***

Bokoch, G.M., Katada, T., Northup, J.K., Hewlett, E.L. & Gilman, A.G. (1983). *J. Biol. Chem.* ***258, 2072.***

Bonifacino, J.S. & Dufau, M.L. (1985). *Endocrinology* ***116, 1610.***

Boockfor, F.R. & Schwarz, L.K. (1991). *Endocrinology* ***129, 256.***

Borges, J.L.C., Scott, D., Kaiser, D.L., Evans, W.S. & Thorner, M.O. (1983). *Endocrinology* ***113, 557.***

Borgmann, V., Hardt, W., Schmidt-Gollwitzer, M., Adenauer, H. & Nagel, R. (1982). *Lancet* ***1, 1097.***

Bortolussi, M., Zanchetta, R., Belvedere, P. & Colombo, L. (1990). *Cell Tissue Res.* ***260, 185.***

Bouin, P. & Ancel, P. (1903). *Archives de Zoology (Stockh) [serie IV]* ***1, 437.***

Bouin, P. & Ancel, P. (1905). *Archives de Zoology Experimentale Gen* ***3, 391.***

Bourguignon, J.P., Frachimont, P. (1984). *Endocrinology* ***114, 1941.***

Bourne, G.A., Regiani, S., Payne, A.H. & Marshall (1980). *J. Clin. Endocrinol. Metab.* ***51, 407.***

Bourne, G.A., Dockrill, M.R., Regiani, S., Marshall, J.C. & Payne, A.H. (1982). *Endocrinology* ***110, 727.***

Bourne, H.R. (1987). *Nature* ***330, 517.***

Bouvier, C., Forget, H., Lagacé, G., Drews, R., Sinnett, D., Labuda, D. & Collu, R. (1991)a. *Mol. Cell. Endocrinol.* ***78, 33.***

Bouvier, C., Lagacé, G. & Collu, R. (1991)b. *Mol. Cell. Endocrinol.* ***79, 65.***

Braden, T.D., Farnworth, P.G., Burger, H.G. & Conn, P.M. (1990). *Endocrinology* ***127, 2387.***

Bradford, M.M. (1976). *Anal. Biochem.* ***72, 248.***

Bradley, S.F., Vibhagool, A., Kundel, S.L., Kauffman, C.A. (1989). *J. Leuk. Biol.* ***45, 510.***

Brandt, M.E., Puett, D., Garola, R., Fendl, K., Covey, D.F. & Zimniski, S.J. (1988). *In Hormones, cell Biology, and cancer: perspectives and potentials, pp 65-84. Alam R. Liss. Inc.*

Brann, D.W., Putnam, C.D. & Mahesh, V.B. (1990). *Endocrinology **126, 159.***

Brannen, A. L. & Chandler, D. B. (1988). *Am. J. Pathol. **132, 161.***

Brimblecombe, R.W., Leslie, G.B. (1984). *In Safety testing of new drugs, pp 65-91, D.R. Laurence, A.E.M. McLean, M. Weatherall (eds). Academic Press.*

Brinkmann, A.O., Mulder, E., Lamers-stahlhofen, G.J.M., Mechielsen, M.J. & van der Molen, H.J. (1972). *FEBS Letters **26, 301.***

Brinkmann, A.O., Leemborg, F.G., Roodnat, E.M., de Jong, F.H. & van der Molen, H.J. (1980). *Biol. Reprod. **23, 801.***

Brodie, H.J., Kripalani, K.J. & Possanza, G. (1969). *J. Am. Chem. Soc. **91,** 1241.*

Brodie, A.M.H., Schwarzel, W.C. & Brodie, H.J. (1976). *J. Steroid Biochem. 7, 787.*

Brodie, A.M.H., Schwarzel, W.C., Shaikh, A.A. & Brodie, H.J. (1977). *Endocrinology **100, 1684.***

Brooker, G., Harper, J.F., Terasaki, W.L. & Moylan, R.D. (1979). *In: Advances in cyclic nucleotide research, Vol 10, pp 1-33. G. Brooker, P. Greegard & G.A. Robinson (eds). Raven Press: New York.*

Brown, E.M., Carrol, R.J. & Aurbach, G.D. (1977). *Proc Natl. Acad. Sci. USA 74, 4210.*

Brown, C.E., Warren, S., Chute, R.N., Ryan, K.J. & Todd, R.B. (1979). *Cancer Res. **39, 3971.***

Browne, E. S., Flasch, M. V., Sohal, G. S. & Balla, V. K. (1990). *Mol. Cell. Endocrinol. **70, 49.***

Browning, J.Y., D'Agata, R., Steinberger, A., Grotjan, H.E. & Steinberger, E. (1983)a. *Endocrinology **113, 985.***

Browning, J. Y., Heindel, J. J. & Grotjan, H. E. (1983)b. *Endocrinology **112,** 543.*

Bulmer, D. (1964). *J. Anatomy (London)* ***98, 313.***

Bulmer, J.N. & Johnson, P.M. (1984). *Clin. Exp. Immunol.* ***57, 393.***

Bunzow, J.R., van Tol, H.H.M., Grandy, D.K., Albert, P., Salon, J., Christie, M.-D., Machida, C.A., Neve, K.A. & Civelli, O. (1988). *Nature* ***336, 783.***

Burrows, H. (1935). *J. Pathol. Bact.* ***41, 218.***

Buch, J.P., Lamb, D.J., Lipshultz, L.I., Smith, R.G. (1988). *Fert. Steril.* ***49,*** *658.*

Calkins, J. H., Guo, H., Sigel. M. M. & Lin, T. (1990). *Biochim. Biophys. Res. Commun.* ***166, 1313.***

Calkins, J. H., Sigel, M. M., Nankin, H. R. & Lin, T. (1988). *Endocrinology,* ***123, 1605.***

Cameron, I.L. (1970). *Texas Reports on Biology and Medicine* ***28, 203.***

Cao, Y.-Q., Sundaram, K., Bardin, C.W., Rivier, J. & Vale, W. (1982). *Int. J. Androl.* ***5, 158.***

Carbone, M. & Levine, A.S. (1990). *Trends Endocrinol. Metab.* ***1, 248.***

Carter, D.A. & Whitehead, S.A. (1981). *J. Endocrinol.* ***91, 439.***

Caspi, E., Wicha, J., Arunachalam, T., Nelson, P., Spiteller, G. (1984). *J. Am. Chem. Soc.* ***106, 7282.***

Castle, W.N. & Richardson, J.R. (1986). *J. Urology* ***136, 1307.***

Catt, K.J., Tsuruhara, T. & Dufau, M.L. (1972). *Biochim. Biophys. Acta* ***279,*** *194.*

Catt, K.J. & Dufau, M.L. (1973). *Nature* ***244, 219.***

Catt, K.J., Harwood, J.P., Aguilera, G. & Dufau, M.L. (1979). *Nature* ***280,109.***

Catt, K.J. & Pierce, J.G. (1986). *In: Reproductive endocrinology, pp 75-114. S.C.C. Yen & R.B. Jaffe (eds). W.B. Saunders Co: Philadelphia.*

Catt, K.J. & Stojilkovic, S.S. (1989). *Trends Endocrinol. Metabol.* ***1, 15.***

Chan, S.W.C., Leathem, J.H., Esashi, T. (1977). *Endocrinology **101, 128.***

Chan, V., Katikineni, M., Davies, T.F. & Catt, K.J. (1981). *Endocrinology **108, 1607.***

Chantaraprateep, P. & Thibier, M. (1978). *J. Endocrinol. **77, 389.***

Charreau, E.H., Calvo, J.C., Nozu, K., Pignataro, O., Catt, K.J. & Dufau, M.L. (1981). *J. Biol. Chem. **256, 12719.***

Charreau, E.H., Calvo, J.C., Tesone, M., de Souza Valle, L.B. & Baranao, J.L. (1978). *J. Biol. Chem. **253, 2504.***

Charreau, E.H., Attramadal, A., Torjesen, P.A., Purvis, K., Calanda, R. & Hansson, V. (1977). *Mol. Cell. Endocrinol. **6, 303.***

Chasalow, F., Marr, H., Haour, F. & Saez, J.M. (1979). *J. Biol. Chem. **254, 5613.***

Chase, D.J. & Payne, A.H. (1983). *Endocrinology **112, 29.***

Chatelain, P.G., Naville, D. & Saez, J.M. (1987). *Biochem. Biophys. Res. Commun. **146, 1009.***

Chaudhary, L.R. & Stocco, D. (1988). *Biochimie **70, 1799.***

Chase, D. J. & Payne, A. H. (1983). *Endocrinology **112, 29-34.***

Chemes, H.E., Rivarola, M.A. & Bergada, C. (1976). *J. Reprod. Fert. **46, 279.***

Chen, S., Besinan, M.J., Sparkes, R.S., Zollman, S., Klisak, I., Mohandes, T., Hall, P.F., Shively, J.E. (1988). *DNA **7, 27.***

Chen, Y.-D., Kraemer, F.B. & Reaven, G.M. (1980). *J. Biol. Chem. **255, 9162.***

Cheung, C.Y. (1983). *Endocrinology **113, 632.***

Childs, G.V., Ellison, D.G., Lorenzen, J.R., Collins, T.J. & Schwartz, N.B. (1982). *Endocrinology **111, 1318.***

Childs, G.V., Hyde, C., Naor, Z., Catt, K. (1983). *Endocrinology **113, 2130.***

Childs, G.V., Unabia, G., Wierman, M.E., Gharib, S.D. & Chin, W.W. (1990). *Endocrinology **126, 2205.***

Chilvers, C.E.D., Pike, M.C. & Peckham, M.J. (1987). *Brit. J. Cancer* ***55, 105.***

Chio, C.L., Hess, G.F., Scott Graham, R. & Huff, R.M. (1990). *Nature* ***343, 266-269.***

Choi, M.S.K. & Cooke, B.A. (1990). *FEBS Letters* ***261, 402.***

Christensen, A.K. (1975). *In: Handbook of physiology, Vol V, pp 57-94. D.W. Hamilton & R.O. Greep (eds). Washington DC: American Physiological Society.*

Christensen, A.K. & Fawcett, D.W. (1966). *Am. J. Anat.* ***118, 551.***

Christensen A.K. & Gillim, S.W. (1969). *In: The gonads, pp 415-488. Ed K.W. McKerns. Appleton-Century-Crofts: New York.*

Christensen, A.K. & Peacock, K.C. (1980). *Biol. reprod.* ***22, 383.***

Chung, B., Matteson, K.J., Miller, W.L. (1986). *Proc. Natl. Acad. Sci. USA.* ***83, 4243.***

Chung, B., Picado-Leonard, J., Haniu, M., Bienkowski, M., Hall, P.F., Shively, J.E. & Miller, W.L. (1987). *Proc. Natl. Acas. Sci. USA* ***84, 407.***

Cigorraga, S.B., Dufau, M.L. & Catt, K.J. (1978). *J. Biol. Chem.* ***253, 4297.***

Cigorraga, S.B., Sorell, S., Bator, J., Catt, K.J., Dufau, M.L. (1980). *J. Clin. Invest.* ***65, 699.***

Ciccarelli, E., Giusti, M., Miola, C., Potenzoni, F., Sghedoni, D., Camanni, F. & Giordano, G. (1989). *J. Clin. Endocrinol. Metab.* ***69, 725.***

Clapper, D.L. & Conn, P.M. (1985). *Biol. Reprod.* ***32, 269.***

Clausen, O. P. F., Purvis, K. & Hansson, V. (1981). *Acta Endocrinol.* ***96, 569.***

Clayton, R.N. (1982). *Endocrinology* ***111, 152.***

Clayton, R.N. (1987). *Clin. Endocrinol.* ***26, 361.***

Clayton, R.N. & Huhtaniemi, I.T. (1982). *Nature* ***299, 56.***

Clayton, R.N., Katikineni, M., Chan, V., Dufau, M.L. & Catt, K.J. (1980). *Proc. Natl. Acad. Sci. USA* ***77, 4459.***

Clayton, R.N., Popkin, R.M. & Fraser, H.M. (1982). *Endocrinology **110**, 1116.*

Colbran, R.J., Garton, A.J., Cordle, S.R. & Yeaman, S.J. (1986). *FEBS Letters **201**, 257.*

Cockroft, S. (1987). *TIBS **12**, 75.*

Cole, P.A. & Robinson, C.H. (1988). *J. Am. Chem. Soc. **110**, 1284.*

Coleman, G.L., Barthold, S.W., Osbaldiston, G.W., Faster, S.J. & Jonas, A.M. (1977). *J. Gerontol. **32**, 258.*

Colletta, A.A. (1990). *Cancer Topics **8**, 18.*

Collu, R., Fraschini, F., Martini, L. (1973). *Prog. Brain Res. **39**, 289.*

Conn, P.M., Tsuruhara, T., Dufau, M. & Catt, K.J. (1977). *Endocrinology **101**, 639.*

Cooke, B.A, de Jong, F.H., van der Molen, H.J. & Rommerts, F.F.G. (1972). *Nature **237**, 255.*

Cooke, B.A. (1990). *Mol. Cell. Endocrinol. **69**, C11.*

Cooke, B.A., Abayasekera, D.R.E. & Rose, M.P. (1990). *In: Activation and desensitization of transducing pathways, Vol H44, pp 99-113. Eds T.M. Konijn, M.D. Houslay & P.J.M. van Haastert. NATO ASI Series: Springer-Verlag Berlin Heidelberg.*

Cooke, B. A., Aldred, L. F., Hunter, M. G., Sullivan, M. H. F. & Dix, C. J. (1983). *J. Steroid Biochem. **19**, 359.*

Cooke, B.A, Dirami, G., Chaudry, L., Choi, M.S.K., Abayasekara, D.R.E. & Phipp, L. (1991). *J. Steroid Biochem. Molec. Biol. **40**, 465.*

Cooke, B.A., Dix, C.J., Haberfield, A.D. & Sullivan, M.H.F. (1986). *Horm. Cell Regul. **139**, 111.*

Cooke, B.A., Lindh, L.M. & Janszen, F.H.A. (1976). *Biochem. J. **160**, 439.*

Cooke, B.A., Lindh, L.M., Janszen, F.H.A., van Driel, M.J.A., Bakker, C.P., van der Plank, M.P.I. & van der Molen (1979). *Biochim. Biophys. Acta **583**, 320.*

Cooke, B. A., Magee-Brown, R., Golding, M. & Dix, C. J. (1981). *Int. J. Androl. 4, 355.*

Cooke, B.A. & Rommerts, F.F.G. (1988). *In: Hormones and their actions, Part II, pp 155-162. B.A. Cooke, R.J.B. King & H.J. van der Molen (eds). Elsevier: Amsterdam/ New York/ Oxford.*

Cooke, B.A. & Sullivan, M.H.F. (1985). *Mol. Cell. Endocrinol. 41, 115.*

Corbin, A. & Bex, F.J. (1980). *In: Regulation of male fertility, pp 55-63. G.R. Cunningham, W. -B. Schill & E.S.R. Hafez (eds). Martinus Nijhoff Publishers bv: The Hague/ Boston/ London.*

Corbin, C.J., Graham-Lorence, S., McPaul, M., Mason, J.I., Mendelson, C.R. & Simpson, E.R. (1988). *Proc. Natl. Acad. Sci. USA 85, 8948.*

Corrie, D., Norbeck, J.C., Thompson, I.M., Rodriguey, F., Teague, J.L., Rounder, J.B. & Spence, C.R. (1987). *J. Urology 137, 747.*

Corrigan, A.Z., Bilezikjian, L.M., Carroll, R.S., Bald, L.N., Schmelzer, C.H., Fensly, B.M., Mason, A.J., Chin, W.W., Schwall, R.H., Vale, W. (1991). *Endocrinology 128, 1682.*

Costlow, M.E., McGuire, W.L. (1977). *J. Endocrinol. 75, 221.*

Cote, T.E., Grewe, C.W. & Kebabian, J.W. (1981). *Endocrinology 108, 420.*

Courrier, R. (1966). *Actualités Endocrinologiques (Paris) 7, 11.*

Cozza, E.N., Carmen Vila, M.D., Acevedo-Duncan, M., Gomez-Sanchez, C.E. & Farese, R.V. (1990). *J. Steroid Biochem. 35, 343.*

Crowley, W.R., Hassid, A., Kalra, S.P. (1987). *Endocrinology 120, 941.*

Culler, M.D. & Negro-Vilar, A. (1990). *Mol. Cell. Endocrinol. 70, 89.*

Cumming, D.C., Quigley, M.E., Yen, S.S.C. (1983). *J. Clin. Endocrinol. Metab. 57, 671.*

Dalgaard, J.B. & Hesselberg, F. (1957). Acta. Pathol. Microbiol. Scand. **41**, 219.

Dalkin, A.C., Haisenleder, D.J., Ortolano, G.A., Ellis, T.R. & Marhall, J.C. (1989). *Endocrinology 125, 917.*

Dan-Cohen, H. & Naor, Z. (1990). *Mol. Cell. Endocrinol.* ***69, 135.***

Dangott, L.J., Puett, D., Gaebers, D.L. & Melner, M.H. (1983). *Biochim. Biophys. Res. Commun.* ***116, 400.***

Dangott, L.J., Puett, D. & Melner, M.H. (1986). *Biochim. Biophys. Acta* ***886,*** *187.*

Dannies, P.S. & Rudnick, M.S. (1980). *J. Biol. Chem.* ***225, 2776.***

Davidson, J., van der Merwe, P.A., Wakefield, I. & Millar, R.P. (1991). *Mol. Cell. Endocrinol.* ***76, C33.***

Davila, D.R., Edwards III, C.K., Arkins, S., Simon, J., Kelly, K.W. (1990). *FASEB J.* ***4, 2906.***

Davis, V., DiMartino, N.A. & Schneider, G. (1980). *J. Clin. Endocrinol. Metabol.* ***51, 1293.***

De Camilli, P. & Macconi, D. (1979). *Nature* ***278, 252.***

Dehejia, A., Nozu, K., Catt, K.J. & Dufau, M.L. (1982). *J. Biol. Chem.* ***257,*** *13781.*

de Jong, F.H. (1974). *PhD Thesis, Department of Biochemistry, Erasmus University, Rotterdam, The netherlands.*

de Jong, F.H., Grootenhuis, A.J., Steenbergen, J., van Sluijs, F.J., Foekens, J.A., ten Kate F.J.W., Oosterhuis, J.W., Lamberts, S.W.J. & Klijn J.G.M. (1990) *J. Steroid Biochem.* ***37, 863.***

de Jong, F.H., Hey, A.H. & van der Molen, H.J. (1973). *J. Endocrinol.* ***57, 277.***

de Jong, F.H., Hey, A.H. & van der Molen, H.J. (1974). *J. Endocrinol.* ***60, 409.***

de Kretzer, D.M. (1982). *Int. J. Androl. (Suppl.)* ***5,*** *11.*

de Kretzer, D.M. & Kerr, J.B. (1988). *In: The physiology of reproduction, pp 837-932. E. Knobil, J.D. Neill, L.L. Ewing, G.S. Greenwald, C. Markert & D.W. Pfaff. Raven Press Ltd: New York.*

de Kretzer, D.M., Sharpe, R.M. & Swanston, I.A. (1979). *Endocrinology* ***105,*** *135.*

Delaage, M.A., Roux, D. & Cailla, H.L. (1979). *In: Advances in Pharmacology and therapeutics: ions-cyclic nucleotides-cholinergy, Vol 3, pp 193-205. J.C. Stoclet (ed). Pergamon Press: Oxford/New york.*

de Leo, V., Petraglia, F., Bruno, M.G., Lanzetta, D., Inaudi, P. & D'Antona, N. (1989). *Gynecol. Obstet. Invest. **27, 94.***

Delidow, B.C., White, B.A. & Peluso, J.J. (1990). *Endocrinology **126, 2302.***

Dell, K.R. & Severson, D.L. (1989). Effect of Cis-unsaturated fatty acids on aortic protein kinase activity. *Biochem. J. **258, 171.***

de Marinis, L., Mancini, A., Calabro, F., Massori, M., Torlontano, M. & Barbarino, A. (1983). *Acta Endocrinol. **10, 385.***

Denef, C. (1988). *In: Hormones and their actions, Part II, pp 113-134. B.A Cooke, R.J.B. King and H.J. van der Molen (eds). Amsterdam/ New York/ Oxford: Elsevier.*

Dennis E.A., Rhee, S.G., Billah, M.M. & Hannun, Y.A. (1991). *FASEB J. **5, 2068.***

Desjardins, C. & Ewing, L.L. (1971). Testicular metabolism in adrenalectomized and corticosterone-treated rats. *Proc. Soc. Exp. Biol. Med. **137, 578.***

Didolkar, A.K. & Sundaram, K. (1987). *Life Sciences **41, 471.***

Dijkstra, C. D., Döpp, E. A., Jolling, P. & Kraal, J. (1985). *Immunology **54, 589.***

Dinarello, C.A. (1984). *Review of infectious diseases **6, 51.***

Dix, C.J., Schumacher, M. & Cooke, B.A (1982). *Biochem. J. **202, 739.***

Dix, C. J., Habberfield, A. D., Sullivan, M. H. F. & Cooke, B. A. (1984). *Biochem. J. **219, 529.***

Dixon, F.J. & Moore, R.A. (1953). *Cancer **6, 427.***

Dluzen, D.E. & Ramirez, V.D. (1987). *Neuroendocrinology **45, 328.***

Dodson, W.C. & Schomberg, D.W. (1987) *Endocrinology **120, 512.***

Doerr, P. & Pirke, K.M. (1976). *J. Clin. Endocrinol. Metab. **43, 622.***

Doerr, P. & Pirke, K.M. (1979). *J. Steroid Biochem. **10, 81.***

Dohlman, H.G., Caron, M.G. & Lefkowitz, R.J. (1987). *Biochemistry **26, 2657.***

Donald, R.A., Wheeler, M., Sönksen, P.H. & Lowy, C. (1983). *Clin. Endocrinol. **18, 385.***

Dorrington, J.H. & Armstrong, D.T. (1975). *Proc. Natl. Acad. Sci. USA **72,** 2677.*

Dorrington, J.H. & Armstrong, D.T. (1979). *Rec. Prog. Horm. Res. **35, 301.***

Dorrington, J.H. & Fritz, I.B. (1975). *Endocrinology **96, 879.***

Drosdowsky, M., Menon, K.M.J., Forchielli, E., and Dorfman, R.I. (1965). *Biochim. Biophys. Acta **104, 229.***

Drouva, S.V. & Gallo, R.V. (1976). *Endocrinology **99, 651.***

Drummond, A.E., Risbridger, G.P., O'Leary, P.C. & de Kretzer, D.M. (1988). *J. Reprod. Fert. **83, 141.***

Drummond, A.E., Risbridger, G.P. & de Kretzer, D.M. (1989). *Endocrinology **125, 510.***

Dubey, A.K. & Plant, T.M. (1985). *Biol. Reprod. **33, 423.***

Dufau, M.L. & Catt, K.J. (1978). *Methods Enzymol. **39D, 252.***

Dufau, M.L., Catt, K.J. & Tsuruhara, T. (1971). *Biochim. Biophys. Acta **252,** 574.*

Dufau, M.L., Cigorraga, S.B., Baukal, A.J., Bator, J.M., Sorrell, S.H., Neubauer, J.F. & Catt, K.J. (1979)a. *J. Steroid Biochem. **11, 193.***

Dufau, M.L., Cigorraga, S., Baukal, A.J., Sorrell, S., Bator, J.M., Neubauer, J.F. & Catt, K.J. (1979)b. *Endocrinology **105, 1314.***

Dufau, M.L., Horner, K.A., Hayashi, K., Tsuruhara, T., Conn, P.M. & Catt, K.J. (1978). *J. Biol. Chem. **253, 3721.***

Dufau, M.L., Watanabe, K. & Catt, K.J. (1973). *Endocrinology **92, 6.***

Dumont, J.E., Jauniaux, J.-C. & Roger, P.P. (1989). *TIBS **14, 67.***

Dunn, M.G. & Bosmann, H.B. (1981). *Biochim. Biophys. Res. Comm. **99, 1081.***

Dupont, E., Luu-The, V., Labrie, F. & Pelletier, G. (1990). *Mol. Cell. Endocrinol.* ***74, R7.***

Dupont, E., Zhao, H.-F., Rhéaume, E., Simard, J., Luu-The, V., Labrie, F. & Pelletier, G. (1990). *Endocrinology* ***127, 1394.***

Du Ruisseau, P., Tache, Y., Brazeau, P., Collu, R. (1978). *Neuroendocrinology* ***27, 257.***

Earp, H.S., Rubin, R.A., Austin, K.S. & Dy, R.C. (1983). *Biochim. Biophys. Res. Commun.* ***112, 413.***

Eccles, S.A, Alexander, P. (1974). *Nature* ***250, 667.***

Edman, C.D., Mac Donald, P.C. (1978). *Am. J. Obst. Gynecol.* ***130, 456.***

Edwards III, C.K., Lorence, R.M., Dunham, D.M., Arkins, S., Yunger, L.M., Greager, J.A., Walter, R.J., Dantzer, R. & Kelly, K.W. (1991). *Endocrinology* ***128, 989.***

Edwards, G., Lendon, R. & Morris, I.D. (1989). *J. Endocrinol.* ***123, 197.***

EK, B. & Heldin, C.-H. (1982). J. Biol. Chem. **257**, 10486.

El-Demiry, M.I., Hargreave, T.B., Busuttil, A., Elton, R., James, K. & Chisholm, G.D. (1987). *Fert. Steril.* ***48, 470.***

Emanuale, N.V., Emanuele, M.A., Tentler, J., Kirsteins, L., Azad, N. & Lawrence, A.M. (1990). *Endocrinology* ***126, 2482.***

Enz, A. (1981). *Life Science* ***29, 2227.***

Enz, A., Donatsch, P., Nordmann, R. (1984). *J. Neural. Transmission* ***60, 225.***

Erichsen, A., Jahnsen, T., Andersen, D., Torjesen, P. & Hansson, V. (1984). *J. Steroid Biochem.* ***21, 539.***

Erickson, G.F., Hsueh, A.J.W. (1978). *Endocrinology* ***102, 1275.***

Erickson, G.F., Garzo, V.G. & Magoffin, D.A. (1989). *J. Clin. Endocrinol. Metabol.* ***69, 716.***

Erickson, L.D., Rizza, S.A., Bergert, E.R., Charlesworth, M.C., McCormick, D.J. & Ryan, R.J. (1990). *Endocrinology* ***126, 2555.***

Eskeland, N.L., Lugo, D.I., Pintar, J.E. & Schachter, B.S. (1989). *Endocrinology* ***124, 2914.***

Evans, C.T., JoCorbin, C., Saunders, C., Merrill, J.C., Simpson, E.R. & Mendelson, C.R. (1987). *J. Biol. Chem.* ***262, 6914.***

Evans, J.A., Robinson, G., Catt, K.J. (1989). *J. Endocrinol.* ***122, 99.***

Evans, R. (1973). *Br. J. Cancer* ***28 (Suppl 1), 19.***

Ewing, L.L., Zirkin, B.R. (1983). *Rec. Prog. Horm. Res.* ***39, 599.***

Ewing, L.L., Zirkin, B.R., Cochran, R.C., Fromann, N., Peters, C. & Ruitz-Bravo, N. (1979). *Endocrinology* ***105, 1135.***

Ewing, L.L., Wing, T.-Y., Cochran, R.C., Kromann, N. & Zirkin, B.R. (1983). *Endocrinology* ***112, 1763.***

Fabbri, A. (1990). *Trends Endocrinol. Metab.* ***1, 117.***

Fabbri, A., Knox, G., Buczko, E. & Dufau, M.L. (1988). *Endocrinology* ***122, 749.***

Fabbri, A., Tinajero, J.C. & Dufau, M.L. (1990). *Endocrinology* ***127, 1541.***

Faure, N., Labrie, F., Lemay, A., Bélanger, A., Gourdeau, Y., Laroche, B. & Robert, G. (1982). *Fert. Steril.* ***37, 416.***

Fauser, B.C.J.M., Baird, A. & Hsueh, A.J.W. (1988)a. *Endocrinology* ***123, 2935.***

Fauser, B. C. J. M., Galway, B. & Hsueh, A. J. W. (1988)b. *In: The molecular and cellular endocrinology of the testis. Vol. 50, pp. 281-295. B.A. Cooke & R.M. Sharpe (eds). Raven Press: New York.*

Fave, G.F.D., Tamburrano, G., de Magistris, L., Natoli, C., Sautoro, M.L., Carrutu, R. & Torsoli, A. (1977). *Lancet* ***1, 1319.***

Fawcett, D.M., Neaves, W.B. & Flores, M.N. (1973). *Biol. Reprod.* ***9, 500.***

Ferrari, C., Rampini, P., Malinverni, A., Scarduelli, C., Benco, R., Caldara, R., Barbieri, C., Testori, G. & Crosignani, P.G. (1981). *Acta Endocrinol.* ***97, 436.***

Ferrari, C., Barbieri, C., Caldara, R., Mucci, M., Codecasa, F., Paracchi, A., Romano, C., Boghem, M. & Dubin, A. (1986). *J. Clin. Endocrinol. Metab.* ***63,*** *941.*

Fevold, R.H., Lorence, M.C., MarCarthy, J.L., Trant, J.M., Kagimoto, M., Waterman, M.R. & Masson, J.I. (1989). *Mol. Endocrinol.* ***3, 968.***

Fidler, I.J. (1976). *In: The macrophage in neoplasia, pp 245-257. Academic Press: New York; San francisco; London.*

Fink, G. (1979). *Br Med Bulletin* ***35, 155.***

Fisher, L.A. (1989). *TIPS* ***10, 189.***

Fishman, J. & Goto, J. (1981). *J. Biol. Chem.* ***256, 4466.***

Fishman, J. & Raju, M.S. (1981). *J. Biol. Chem.* ***256, 4472.***

Flower, R.J. (1988). *Brit. J. Pharmacol.* ***94, 987.***

Flückiger, E., Richardson, B.P., Donatsch, P. & Enz, A. (1983). *In: Aging Vol. 23: Aging brain and ergot alkaloids, A. Agnoli et al. (eds). Raven Press: New York.*

Folman, Y., Ahmad, N., Sowell, J.G. & Eik-Nes, K.B. (1973). *Endocrinology* ***92, 41.***

Forchielli, E. & Dorfman, R.I. (1956). *J. Biol. Chem.* ***223, 443.***

Forest, M.G., Lecoque, A. & Saez, J.M. (1979). *J. Clin. Endocrinol. Metab.* ***49, 284.***

Fouquet, J.-P. & Kann, M.-L. (1987). *Reprod. Nutr. Develop.* ***27, 1053.***

Fournet-Dulguerov, N., MacLusky, N.J., Leranth, C.Z., Todd, R., Mendelson, C.R., Simpson, E.R., Naftolin, F. (1987). *J. Clin. Endocrinol. Metab.* ***65, 757.***

Fox, S.E. & Smith, M.M.S. (1985). *Endocrinology* ***116, 1485.***

Franklyn, J.A. & Sheppard, M.C. (1989). *Trends Endocrinol. Metab.* ***1, 35.***

Franks, S., Horrocks, P.M., Lynch, S.S., Butt, E.R. & London, D.R. (1981). *Lancet* ***2, 659.***

Fraser, R., Connell, J.M., Inglis, G., Kenyon, C.J., Tree, M. (1989). *J. Steroid Biochemistry* ***32, 217.***

Freeman, D.A. (1987)a. *J. Biol. Chem.* ***262, 13061.***

Freeman, D.A. (1987)b. *Endocrinology* ***120, 124.***

Freeman, D.A. (1989). *Endocrinology **124**, 2527.*

Freeman, D.A. & Ascoli, M. (1981). *Proc. Natl. Acad. Sci. USA **78**, 6309.*

Freeman, D.A. & Ascoli, M. (1982)a. *Proc. Natl. Acad. Sci. USA **79**, 7796.*

Freeman, D.A. & Ascoli, M. (1982)b. *J. Biol. Chem. **257**, 14231.*

Fritz, I.B., Griswold, M.D., Louis, B.F., Dorrington, J.H. (1976). *Mol. Cell. Endocrinol. **5**, 289.*

Frφysa, A., Ascoli, M., Segaloff, D., Beebe, S., Jahnsen, T. & Hansson, V. (1988). *In: The molecular and cellular endocrinology of the testis, Vol. 50, pp 65-72. B.A. Cooke & R.M. Sharpe (eds). Serono Symposia Publications: Raven Press.*

Fujimoto, M., Berkovitz, G.D., Brown, T.R. & Migeon, C.J. (1986). *J. Clin. Endocrinol. Metab. **63**, 468.*

Fuller, P.J. (1988). *Mol. Cell. Endocrinol. **59**, 161.*

Funder, G.W. & Mercer, J.E. (1979). *J. Clin. Endocrinol. Metab. **48**, 189.*

Gabrilove, J.L., Nicolis, G.L., Mitty, H.A. & A.R. Sohval (1975). *Cancer **35**, 1184.*

Gabrilove, J.L., Nicolis, G.L. & Sohval, A.R. (1979). *J. Urology **112**, 95.*

Gaillard, R.C., Abeywickrama, K., Brownell, J. & Muller, A.F. (1989). *J. Clin. Endocrinol. Metab. **68**, 329.*

Gale, J.S., Wakefield, J. St.J. & Ford, H.C. (1982). *J. Endocrinol. **92**, 293.*

Gallagher, T.F., Kock, F.C. (1929). *J. Biol. Chem. **84**, 495.*

Garner, L.L., Campbell, G.T. & Blake, C.A. (1990). *Endocrinology **126**, 992.*

Gavish, M., Okun, F., Weizman, A. & Youdim, M.B.H. (1986). *Eur. J. Pharmacol. **127**, 147.*

Gayton, F., Bellido, C., Aceitero, J., Aguilar, E. & Sancher-criado, J.E. (1990). *Biol. Reprod. **47**, 665.*

Gelberg, H.B. & McEntee, K. (1987). *Veterinary Pathology **24**, 231.*

Genton, C.Y. (1980). *Arch. Gynaek. **230**, 49.*

Ghanadian, R. & Puah, C.M. (1981). *J. Endocrinol.* ***88, 255.***

Gharib, S.D., Wierman, M.E., Badger, T.M. & Chin, W.W. (1987). *J. Clin. Invest.* ***80, 292.***

Gilman, A.G. (1984). G proteins and dual control of adenylate cyclase. *Cell* ***36,*** *577.*

Giros, B., Sokoloff, P., Martiesm M.-P., Riou, J.-F., Emorine, L.J. & Schwartz, J.-C. (1989). *Nature* ***342, 923.***

Gitay-Goren, H., Lindenbaumn E.S. & Kraiemn Z. (1989). *Mol. Cell. Endocrinol.* ***65, 195.***

Gitelman, S.E. (1990). *Trends Endocrinol. Metab.* ***1, 181.***

Goldberg, B., Jones, G.E.S., Borkowf, H.J. (1964). *J. Histochem. Cytochem.* ***12,*** *880.*

Goldsmith, P.C., Cronin, M.J., Weiner, R.I. (1979). *J. Histochem. Cytochem.* *27, 1205.*

Gondos, B. (1977). Testicular development. *In: The testis. Advance in physiology, biochemistry and function, vol 4, pp1-37. Ed A.D. Johnson & WR Gomes. New York: Academic Press.*

Gonzaley-Mancho, C. & Vale, W. (1989). *Endocrinology* ***125, 1666-1672.***

Gordon, W.L. & Ward, D.N. (1985). *In: Structural aspects of luteinizing hormone action and receptors, pp 173-197. CRC Press, Boca Raton, FL Raton.*

Goto, J. & Fishman, J. (1977). *Science* ***80, 195.***

Gower, D.B. (1988). In: Hormones and their actions, Part I, pp3-28. B.A. Cooke, R.J.B. King & H.J. van der Molen (eds). Elsevier.

Grandy, D.K., Marchionni, M.A.M Makam, H., Stofko, R.E., Alfano, M., Frothingham, L., Fisher, J.B., Burke-Howie, K.J., Bunzow, J.R., Server, A.C. & Civelli, O. (1989). *Proc. Natl. Acad. Sci. USA* ***86,*** *9762.*

Gray, G.D., Smith, E.R., Damassa, D.A., Ehrenkranz, J.R.L. & Davidson, J.M. (1978). *Neuroendocrinology* ***25,*** *247.*

Greep, R.O. (1937). *Cold Spring Harbor Symposia Quant. Biol.* ***5,*** *136.*

Greep, R.O., Fevold, H.L. & Hisaw, F.L. (1936). *Anat. Rec.* ***65,*** *261.*

Greenberg, M.E. & Ziff, E.B. (1984). *Nature* ***311, 433.***

Grem, J.L., Robins, H.I., Wilson, K.S., Gilchrist, K. & Trump, D.L. (1986). *Cancer* ***58, 2116.***

Grodin, J.M., Siiteri, P.K., MacDonald, P.C. (1973). *J. Clin. Endocrinol. Metab.* ***36, 207.***

Grootenhuis, A.J., van Sluijs, F.J., Klaij, I.A., Steenbergen, J., Timmerman, M.A., Bevers, M.M., Dieleman, S.J. & de Jong, F.H. (1990)a. *J. Endocrinol.* ***127, 235.***

Grootenhuis, A.J., van Beurden, W.M.O., Timmerman, M.A. & de Jong, F.H. (1990). *Mol. Cell. Endocrinol.* ***74, 125.***

Gross, J.L., Krupp, M.N., Rifkin, D.B., Lane, M.D. (1983). *Proc. Natl. Acad. Sci. USA* ***80, 2276.***

Guo, H., Harrington, J., Sigel, M.M. & Lin, T. (1990). *Endocrinology* ***127,1234.***

Gupta, D., Rager, K., Zarzycki, J. & Eichner, M. (1975). *J. Endocrinol.* ***66,*** *183.*

Habberfield, A.D., Dix, C.J. & Cooke, B.A. (1986). *Biochem. J.* ***233, 369.***

Habberfield, A.D. & Cooke, B.A. (1987). *J. Endocrinol.* ***114, 415.***

Hafiez, A.A., Bartke, A. & Lloyd, C.W. (1972). *J. Endocrinol.* ***53, 223.***

Hahn, E.F. & Fishman, J. (1984). *J Bio. Chem.* ***259, 1689.***

Haider, S.G., Pickards, H., Freundl, G. & Passia, D. (1985). *Acta Anatomica* ***121, 170.***

Haisenleder, D.J., Dalkin, A.C., Ortolano, G.A., Marshall, J.C. & Shupnik, M.A. (1991). *Endocrinology* ***128, 509.***

Hales, D.B. & Payne, A.H. (1989). *Endocrinology* ***124, 2099.***

Hales, D.B., Sha, L. & Payne, A.H. (1987). *J. Biol. Chem.* ***262, 11200.***

Hall, P.F., Irby, D.C., de Kretzer, D.M. (1969). *Endocrinology* ***84, 488.***

Hall, P.F., Charponnier, C., Nakamura, M. & Gabbiani, G. (1979). *J. Steroid Biochem.* ***11, 1361.***

Hall, P.F., Osawa, S. & Mrotek, J. (1981). *Endocrinology **109, 1677.***

Hall, S.H., Joseph, D.R., French, F.S. & Conti, M. (1988). *Mol. Endocrinol. 2, 55.*

Hallberg, M.C., Wieland, R.G., Zoin, E.M., Furst, B.H., Wieland, J.M. (1976). *Fert. Steril. 27, 812.*

Hamernik, D.K. & Nett, T.M. (1988). *Endocrinology **122, 959.***

Haour, F., Bouznetzova, B., Dray, F. & Saez, J.M. (1979). Life Sci. **24**, 2151.

Haour, F. & Saez, J.M. (1977). *Mol. Cell. Endocrinol. 7, 17.*

Haour, F., Mather, J., Kouznetzova, B. & Dray, F. (1980). *62nd Endocrinology Meeting, June 18-20, Abst 46.*

Harcourt, A.H., Harvey, P.H., Larson, S.G., Short R.V. (1981). *Nature **293, 55.***

Hardie, D.G., Carling, D. & Sim, A.T.R. (1989). *TIBS **14, 20.***

Hardy, M.P., Zirkin, B.R. & Ewing, L.L. (1989). *Endocrinology **124, 762.***

Hardy, M.P., Kelce, W.R., Klinefelter, G.R. & Ewing, L.L. (1990). *Endocrinology **127, 488.***

Harman, S.M., Danner, R.L., Roth, G.S. (1978). *Endocrinology **102, 540.***

Hatzfeld, J., Miskin, R., Reich, E. (1982). *J. Cell Biol **92, 176.***

Hausdorff, W.P., Caron, M.G. & Lefkowitz, R.J. (1990). *FASEB Journal **4, 2881.***

Hedger, M.P. & Eddy, E.M. (1986). *Biol. Reprod. **35, 1309.***

Hedger, M. P. & Eddy, E. M. (1987). *Endocrinology **121, 1824.***

Heller, C.G., Lalli, M.F., Pearson, J.E. & Leach, D.R. (1971). *J. Reprod. Fert. 25, 177.*

Heller, C.G. & Leach, D.R. (1971). *J. Reprod. Fert. **25, 185.***

Hikim, A.P.S., Amador, A.G., Bartke, A. & Russell, L.D. (1989). *Endocrinology **125, 1844.***

Hodson, C.A., Simkins, J.W., Aylsworth, C.F., Steger, R.W. & Meites, J. (1980). *Neuroendocrinology **30, 7.***

Hodgson, Y. & Hudson, B. (1983). *Monographs on Endocrinology* ***7, 107.***

Hoffman, Y.M., Peegel, H., Sprock, M.J.E., Zhang, Q.-Y. & Menon, K.M.J. (1991). *Endocrinology* ***128, 388.***

Holmes, S.D., Lipshultz, L.I. & Smith, R.G. (1984). *J. Clin. Endocrinol. Metab.* ***59, 1058.***

Holmes, S.D., Spotts, G. & Smith, R.G. (1986). *J. Biological Chem.* ***261, 4076.***

Holz IV, G.G, Rane, S.G. & Dunlap. K. (1986). *Nature* ***319, 670.***

Hou, J.W., Collins, D.C. & Schleicher, R.L. (1990). *Endocrinology* ***127, 2047.***

Houben, H. & Denef, C. (1990). *Trends Endocrinol. Metab.* ***1, 398.***

Hsueh, A.J.W., Dufau, M.L. & Catt, K.J. (1978). *Endocrinology* ***103, 1096.***

Hsueh, A.J.W., Bambino, T.H., Zhuang, L.-Z., Welsh, T.H. & Ling, N.C. (1983). *Endocrinology* ***112, 1653.***

Hsueh, A.J., Dahl, K.D., Vaughan, J., Tucker, E., Rivier, J., Bardin, C.W. & Vale, W. (1987). *Proc. Natl. Acad. Sci. USA* ***84, 5082.***

Hsueh, A.J.W. & Erickson, G.F. (1979). *Nature* ***281, 66.***

Hsueh, A.J.W., Schreiber, J.R. & Erickson, G.F. (1981).
Mol. Cell. Endocrinol. ***21, 43.***

Huet-Hudson, Y.M., Andrews, G.K. & Dey, S.K. (1989). *Endocrinology* ***125, 1683.***

Huhtaniemi, I.T. (1983). *In: Clinics in endocrinology and metabolism: receptors in health and disease, Vol 12, pp 117-132. N. Clayton. W.B (ed). Saunders Company Ltd: London-Philadelphia-Toronto.*

Huhtaniemi, I.T., Nozu, K., Warren, D.W., Dufau, M.L. & Catt, K.J. (1982). *Endocrinology* ***111, 1711.***

Huhtaniemi, I.T., Warren, D.W. & Catt, K.J. (1984). *Ann. N.Y. Acad. Sci.* ***438, 283.***

Huhtaniemi, L.T. & Warren, D.W. (1990). *Trends Endocrinol. Metabol.* ***1, 356.***

Hunt, V.C. & Budd, J.W (1939). *J. Urology* ***42, 1242.***

Hunter, M.G. & Cooke, B.A. (1985). *Mol. Cell. Endocrinol.* ***39, 217.***

Hunter, M.G., Sullivan, M., Aldred, L., Dix, C.J. & Cooke, B.A (1982). *Mol. Cell. Endocrinol.* ***27, 31.***

Hunzicker-Dunn, M. & Birnbaumer, L. (1985). *In: Luteinizing hormone action and receptors, pp 57-134. CRC Press: Boca Raton.*

Hurban, Z., Vigil, E.L., Slesers, A., Hopkins, E. (1972). *Lab. Invests* ***27, 184.***

Huseby, R.A. (1981). *Cancer Res.* ***41, 3172.***

Huseby, R.A. & Samuels, L.T. (1977). *J. Natl. Cancer. Inst.* ***58, 1047.***

Hutson, J.C., Yee, J.B. & Yee, J.A. (1987). *Mol. Cell. Endocrinol.* ***52, 11.***

Hutson, J.C. (1989). Leydig cells de not have Fc Receptors. *J. Androl.* ***10, 159.***

Hutson, J.C & Stocoo, D.M. (1989). *Reg. Immunol.* ***2, 249.***

Hutson, J.C. (1990). *Biol. Reprod.* ***43, 885.***

Hwang, J. & Menon, K.M.J. (1984). *J. Biol. Chem.* ***259, 1978.***

Hyde, J.F. & Ben-Jonathan, N. (1989). *Endocrinology* ***125, 736.***

Igorashi, M. (1983). *Int. J. Androl.* ***6, 235.***

Iqubal, M.J., Greenway, B., Wilkinson, M.L., Johnson, P.J. & Williams, R. (1983). *Clin, Sci.* ***65, 71.***

Irvine, R.F. (1982). *Biochemical Journal* ***204, 3.***

Ishi-Ohra, H., Juano, H. & Tamaoki, B. (1986). *J. Steroid Biochem.* ***25, 555.***

Isomaa, V., Parvinen, M., Jänne, O.A. & Bardin, C.W. (1985). *Endocrinology* ***116, 132.***

Issemann, I. & Green, S. (1990). *Nature* ***347, 645.***

Jackson, G.L., Kuehl, D. & Rhim, T.J. (1991). *Biol. Reprod.* ***45, 188.***

Jahnsen, T., Purvis, K., Birnbaumer, L. & Hansson, V. (1980). *Int. J. Androl.* ***3, 396.***

Jakobovits, E.B., Majors, J.E. & Varmus, H.E. (1984). *Cell* ***38, 757.***

Jamal, H., Bretherton-Watt, D., Suda, K., Wang, Z.L., Ghatei, M.A., Williams, S. & Bloom, S.R. (1991). J. Endocrinol. **129** (Suppl.), Abstract 93.

James, N., Evan, G., Gullick, W. & Sikora, K. (1988). *Br. J. Cancer **57, 440.***

Janszen, F. H. A., Cooke, B. A., van Driel, M. J. A. & van der Molen, H. J. (1976). *J. Endocrinol. **70, 345.***

Jarvis, W.D., Judd, A.M. & MacLeod, R.M. (1988). *Endocrinology **123, 2793-2799.***

Jellinger, K. (1982). *J. Neurol. **227, 75.***

Jia, X.-C., Oikawa, M., Bo, M., Tanaka, T., Ny, T., Boime, I. & Hsueh, AJW (1991). *Mol. Endocrinol. **5, 759.***

John, M.E., John, M.C. & Boggaram, V., Simpson, E.R., Waterman, M.R. (1986). *Proc. Natl. Acad. Sci. USA 83, 4715.*

Johnson, G.L. & Dhanasekaran, N. (1989). *Endocr. Rev. **10, 317.***

Johnson, M.H. & Everitt, B.J. (eds) (1984). *In: Essential reproduction, pp35-50. Blakwell Scientific Publications: Boston, Edinburgh, London, Melbourne, and Oxford.*

Johnston, J.O. & Metcalf, B.W. (1984). *In: novel approches to cancer chemotherapy, pp307-328. Academic Press: London.*

Johnston, R.B. (1988). *N. Eng. J. Med. **318, 747.***

Jones, T.M., Fang, V.S., Landau, R.L., Rosenfield, R. (1978). *J. Clin. Endocrinol. Metab. **47, 1368.***

Juniewiez, P.E., Keeney, D.S. & Ewing, L.L. (1988). *Endocrinology **122, 891.***

Judd, S.J., Rakoff, J.S. & Yen, S.S.C. (1978). *J. Clin. Endocrinol. Metab. **47, 494.***

Kaler, L.W. & Neaves, W.B. (1981). *Endocrinology **108, 712.***

Kalra, P.S., Crowley, W.R., Kalra, S.P. (1987). *Endocrinology **120, 178.***

Kalra, S.P. & Kalra, P.S. (1983). *Endocr. Rev. **4, 311.***

Kamel, F., Balz, J.A., Kubajk, C.L., Schneider, V.A. (1987). *Endocrinology **120, 1651.***

Kancheva, L.S., Martinova, Y.S. & Goergiev, V.D. (1990). *Mol. Cell. Endocrinol.* ***69, 121.***

Kaplan, M.R. & Simoni, R.D. (1985). *J. Cell Biol.* ***101, 446.***

Kasson, B.G. & Hsueh, J.W. (1986). *Endocrinology* ***118, 23.***

Kasuga, F., Shiota, K. & Takahashi, M. (1989). *J. Reprod. Fert.* ***87, 179.***

Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L. & Kahn, C.R. (1983). *Proc. Natl. Acad. Sci. USA* ***80, 2137.***

Katada, T., Gilaman, A.G., Watanabe, Y., Baner, S., Jakobs, K.H. (1985). *Eur. J. Biochem.* ***151, 431.***

Katayama, T., Shiota, K. & Takahashi, M. (1990). *Mol. Cell. Endocrinol.* ***69,*** *179.*

Katayama, T., Shiota, K. & Takahashi, M. (1991). *Mol. Cell. Endocrinol.* ***77,*** *167.*

Katikineni, M., Davies, T.F. & Catt, K.J. (1981). *Endocrinology* ***108, 2367.***

Kato, Y., Dupre, J. & Beck, J.C. (1973). *Endocrinology* ***93, 135.***

Kay, S., Fu, Y., Koontz, W.W. & Chen, A.T.L. (1975). *Am. J. Clin. Pathol.* ***63,*** *366.*

Kaynard, A.H. & Spies, H.G. (1991). *Endocrinology* ***128, 2769.***

Kebabian, J.W. & Calne, D.B. (1979). *Nature* ***277, 93.***

Kebabian, J.W., Petzold, G.L. & Greengard, P. (1972). *Proc. Natl. Acad. Sci. USA* ***69, 2145.***

Keel, B.A. & Abney, T.O. (1982). *Biochem. Biophy. Res. Commun.* ***107, 1340.***

Keeney, D.S., Sprando, R.L., Robaire, B., Zirkin, B.R. & Ewing, L.L. (1990). *J. Endocrinol.* ***127, 47.***

Kelch, R.P., Jenner, M.R., Weinstein, R.L., Kaplan, S.L. & Grumbach, M. (1972). *J. Clin. Invest.* ***51, 824.***

Keller, G.A., Scallen, T.J., Clarke, D., Maher, P.A., Krisans, S.K. & Singer, S.J. (1989). *J. Cell Biol.* ***108, 1353.***

Kellokumpu, S. & Rajaniemi, H. (1985). *Endocrinology **116, 707.***

Kelly, K, Cochran, B., Stiles, C. & Leder, P. (1983). *Cell **35, 603.***

Kelly, P.M.A., Davison, R.S., Bliss, E. & McGee, J.O'D. (1988). *Br. J. Cancer **57, 174.***

Kelly, R.W., Deam, S., Cameron, M.J. & Seamark, R.F. (1986). *Prostagl. Leukot. Med. **24, 1.***

Kelly, W.G., Judd, D. & Stolee, A. (1977). *Biochemistry **16, 140-145.***

Kerr, J.B. & Donachie, K. (1986). *Cell Tissue Res. **245, 649.***

Kerr, J.B. & Knell, C.M. (1988). *Development **103, 535.***

Kerr, J.B., Rich, K.A., de Kretzer, D.M. (1979). *Biol. Reprod. **20, 409.***

Kerr, J.B. & Sharpe, R.M. (1985). *Endocrinology **116, 2592.***

Krulich, L (1979). *Ann. Rev. Physiol. **41, 603.***

Keski-Oja, J. & Moses, H.L. (1987). *Med. Biol. **65, 13.***

Ketelslegers, J.-M., Hetzel, W.D., Sherins, R.J. & Catt, K.J. (1978). *Endocrinology **103, 212.***

Keutmann, H.T., McIlroy, P.J., Bergert, E.R. & Ryan, R.J. (1983). *Biochemistry 22, 3067.*

Khalfallah, Y., Claustrat, B., Grochowicki, M., Flocard, F., Horlait, S., Serusclat, P. & Sassolas, G. (1990). *J. Clin. Endocrinol. Metab. **71, 354.***

Khan, S.A. & Nieschlag, E. (1991). *Mol. Cell. Endocrinology **75, 1.***

Khan, S.A., Schmidt, K., Hallin, P., Di Pauli, R., De Geyter, Ch. & Nieschlag, E. (1988). *Mol. Cell. Endocrinology **58, 221.***

Khan, S.A., Teerds, K. & Dorrington, J. (1991). *Biol. Reprod. **44 (Suppl.),** Abstract 539.*

Kikkawa, U. & Nishizuka, Y. (1986). *Ann. Rev. Cell. Biol. **2, 149.***

Kimura & Suzuki (1965). *Biochem. Biophys. Res. Commun. **20, 373.***

Kimura & Suzuki (1967). *J. Biol. Chem. **242, 485.***

Kirsch, T.M., Friwdman, A.C., Vogel, R.L. & Flickinger, G.L. (1981). *Biol. Reprod.* ***25, 629.***

Kitahara, S., Winters, S.J., Attardi, B., Oshima, H. & Troen, P. (1990). *Endocrinology* ***126, 2642.***

Kitajima, N., Chihara, K., Abe, H., Okimura, Y., Fujii, Y., Sato, M., Shakutsui, S., Watanabe, M. & Fujita, T. (1989). *Endocrinology* ***124, 69.***

Klein, G. & Klein, E. (1985). *Nature* ***315, 190.***

Kleinberg, D.L., Lieberman, A., Todd, J., Greising, J., Neophytides, A. & Kupersmith, M. (1980). *J. Clin. Endocrinol. Metab.* ***51, 152.***

Klemcke, H.G., Amador, A.G. & Bartke, A. (1990). *Biol. Reprod.* ***43, 162.***

Klemcke, H.G. & Bartke, A. (1981). *Endocrinology* ***108, 1763.***

Klibanski, A., Shupnik, M.A., Bikkal, H.A., Black, P.M., Kliman, B. & Zervas, N.T. (1988). *J. Clin. Endocrinol. Metab.* ***65, 96.***

Klinefelter, G.R., Hall, P.F. & Ewing, L.L. (1987). *Biol. Reprod.* ***36, 769.***

Knecht, M., Feng, P. & Catt, K.J. (1986). *Biochim. Biophys. Res. Commun.* ***139, 800.***

Kolho, K.-L. & Huhtaniemi, I. (1989). *J. Endocrinol.* ***123, 83.***

Kominami, S., Shinzawa, K. & Takemori, S. (1982). *Biochem. Biophys. Res. Commun.* ***109, 916.***

Kooy, A., Weber, R.F.A., Ooms, M.P. & Vreeburg, J.T.M. (1989). *J. Endocrinol.* ***120, 261.***

Kotsuji, F. Winters, S.J., Attardi, B., Keeping, H.S., Oshima, H. & Troen, P. (1988). *Endocrinology* ***123, 2683.***

Krozowski, Z., Stuchbery, S., White, P., Monder, C. & Funder, J.W. (1990). *Endocrinology* ***127, 3009.***

Krulich, L., Hefco, E., Illner, P., Read, C.B. (1974). *Neudroendocrinology* ***16, 293.***

Krupinski, J., Coussen, F., Bakalyar, H.A., Tang, W.-J., Feinsten, P.G., Orth, K., Slaughter, C., Reed, R.R., Gilman, A.G. (1989). *Science* ***244, 1558.***

Kühn-Velten, N. & Staib, W. (1983). *FEBS Letters* ***154, 70.***

Kuopio, T., Pelliniemi, L.J. & Huhtaniemi, I. (1989)b. *Biol. Reprod.* ***40, 135.***

Kuopio, T., Tapanainen, J., Pelliniemi, L.J. & Huhtaniemi (1989)a. *Development* ***107, 213.***

Kurland, G., Christensen, A.K., Houseby, R.A. (1975). *Cancer Res* ***35, 1671.***

Kwekkeboom, D.J., Hofland, L.J., van Koetsveld, P.M., Singh, R., van den Berge, J.H. & Lamberts, S.W.J. (1990). *J. Clin. Endocrinol. Metabol.* ***71, 718.***

Lachelin, G.C., Leblanc, H., Yen, S.S. (1977). *J. Clin. Endocr. Metab.* ***44, 728.***

Lamb, D.J., Spotts, G.S., Shubhada, S. & Baker, K.R. (1991). *Mol. Cell. Endocrinol.* ***79, 1.***

Lambert, S.W.T. & MacLeod, R.M. (1990). *Physiol. Rev.* ***70, 279.***

Lambeth, J.D., Xu, X.X. & Glover, M. (1987). *J. Biol. Chem.* ***262, 9181.***

Lambeth, J.D., Kitchen, S.E., Farooqui, A.A., Tuckey, R. & Kamin, H. (1982). *J. Biol. Chem.* ***257, 1876.***

Lane, T.A. & Chen, T.T. (1991). *Endocrinology* ***128, 1833.***

Lanoux, M.J., Cleland, W.H., Mendelson, C.R., Carr, B.R. & Simpson, E.R. (1985). *Endocrinology* ***117, 361.***

Lapetina, E.G. (1982). *TIPS* ***3, 115.***

Lapolt, P.S., Jia, X.-C., Sincich, C. & Hsueh, A.T.W. (1991). *Mol. Endocrinol.* ***5, 397.***

Laws, A.O., Kerr, J.B. & de Kretzer, D.M. (1984). *Mol. Cell. Endocrinol.* ***38, 39.***

Laws, A. O., Wreford, M. G. M. & de Kretser, D. M. (1985). *Mol. Cell. Endocrinol.* ***42, 73.***

Lea, T., Smeland, E., Funderud, S., Vaartdal, F., Davises, C., Beiske, K. & Ugelstad, J. (1986). *Scand. J. Immunol.* ***23, 509.***

LeBlanc, H., Lachelin, G.C.L., Abu-Fadil, S. & Yen, S.S.C. (1976). *J. Clin. Endocrinol. Metab.* ***43, 668.***

Leblond, C.P. & Clermont, Y. (1952). *Ann. NY Acad. Sci.* ***55, 548.***

Lebrouille. J.L., Burbach, J.P., de Kloet, E.R., Rommerts, F.F. (1986). Endocrinology **118**, 372.

Lee, V.K.W., de Kretzer, D.M., Hudson, B. & Wang, C. (1975). *J. Reprod. Fert.* ***42, 121.***

Lee, W., Mason, A.J., Schwall, R., Szonyl, E. & Mather, J.P. (1989). *Science* ***243, 396.***

Lee Vance, M.L., Cragun, J.R., Reimnitz, C., Chang, R.J., Rachef, E., Blackwell, R.E., Miller, M.M. & Molitch, M.E. (1989). *J. Clin. Endocrinol. Metab.* ***68, 336.***

Lefévre, A., Finaz, C., Berthelon, M.C. & Saez, J.M. (1985). *Mol. Cell. Endocrinol.* ***40, 107.***

Lefévre, A., Morera, A.M. & Saez, J.M. (1978). *FEBS Lett.* ***89, 287.***

Lefkowitz, R.J., Hausdorff, W.P. & Caron, M.G. (1990). *TIPS* ***11, 190.***

Le Magueresse, B. & Jegou,B. (1986). *Biochem. Biophys. Res. Comm.* ***141, 861.***

Le Magueresse, B. & Jegou, B. (1988)a. *Endocrinology* ***122, 1672.***

Le Magueresse, B. & Jegou, B. (1988)b. *Mol. Cell. Endocrinol.* ***58, 65.***

Leon, M.D., Chiauzzi, V.A., Calvo, J.C., Charreau, E.H. & Chemes, H.E. (1987). *Acta Physiol. Pharmacol. Latinoam* ***37, 277.***

Lephart, E.D., Peterson, K.G., Noble, J.F., George, F.W. & McPhaul, M.J. (1990). *Mol. Cell. Endocrinol.* ***70, 31.***

Lephart, E.D. & Simpson, E.R. (1991) (to be published).

Levinson, P.D., Goldstein, D.S., Munson, P.J., Gill, J.R. & Keiser, H.R. (1985). *J. Clin. Endocrinol. Metab.* ***60, 821.***

Levy, H., Deane, H.W., Ruban, B.L. (1959). *Endocrinology* ***65, 932.***

Leydig, F. (1850). *Z Wiss Zoo* ***2, 1.***

Lin, T. (1985)a. *Endocrinology* ***117, 119.***

Lin, T. (1985)b. *Life Sciences* ***36, 1255.***

Lin, T. Blaisdell, J. & Haskell, J. F. (1987)a. *Biochim. Biophys. Res. Commun.* ***146, 387.***

Lin, T., Blaisdell, J. & Haskell, J.F. (1987)b. *Biochim. Biophys. Res. Commun.* ***149, 852.***

Lin, T., Blaisdell, J., Barbour, K.W. & Thompson, E.A. (1988)a. *Biochem. Biophys. Res. Commun.* ***157, 127.***

Lin, T., Blaisdell, J. & Haskell, J.F. (1988)b. *Endocrinology* ***123, 134.***

Lin, T., Calkins, J.H., Morris, P.L., Vale, W. & Bardin, C.W. (1989). *Endocrinology* ***125, 2134.***

Lin, T., Guo, H., Calkins, J.H., Wang, D. & Chi, R. (1991). *Mol. Cell. Endocrinol.* ***78, 205.***

Lin, T., Haskell, J., Vinson, N. & Terracio, L. (1986). *Endocrinology* ***119, 1641.***

Lin, T., Wang, D., Calkins, G.H., Guo, H., Chi, R. & Housley, P.R. (1990). *Mol. Cell. Endocrinol.* ***73, 147.***

Ling, N., Ying, S.Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. & Guillemin, R. (1986). *Biochim. Biophys. Res. Commun.* ***138, 1129.***

Lipsett, M.B., Sarfaty, G.A., Wilson, H., Wayne Bardin, C. & Fushman, L.M. (1966). *J. Clin. Invest.* ***45, 1700.***

Livingstone, M.S., Sziber, P.P., Quinn, W.G. (1984). *Cell* ***37, 205.***

Lloyd, C.E. & Ascoli, M. (1983). *J. Cell Biol.* ***96, 521.***

Lloyd, J.M. & Childs, G.V. (1988). *Endocrinology* ***122, 1282-1290.***

Lϕnning, P.E., Dowsett, M. & Powles, T.J. (1990). *J. Steroid Biochem.* ***35, 355.***

Loppöhn, R.E., Burger, H.G., Bouma, J., Bangah, M., Krans, M. & de Bruijn, W.A. (1989). *N. Eng. J. Med.* ***321, 790.***

Loosfelt, H., Misrahi, M., Atger, M., Salesse, R., Thi M.T.V.H.L, Jolivet, A., Guiochon-Mantel, A., Sar, S., Jalial, B., Garnier, J., Milgrom, E. (1989). *Science* ***245, 525.***

Lopez-Ruiz, M.P., Choi, M.S.K., Rose, M., West, A.P. & Cooke, B.A. (1992). *Endocrinology (in press).*

Louis, B.G. & Fritz, I.B. (1979). *Endocrinology **104**, 454.*

Loukides, J.A., Loy, R.A., Edwards, R., Honig, J., Visintin, I. & Polan, M.L. (1990). *J. Clin. Endocrinol. Metab. **71**, 1363.*

Lowitt, S., Faresse, R.V., Sabir, M.A. & Root, A.W. (1982). *Endocrinology **111**, 1415.*

Luketich, J.D., Melner, M.H., Guengerich, F.P., Puett, D. (1983). *Biochim. Biophys. Res. Commun. **111**, 424.*

Lyons, J., Landis, C.A., Harsh, G., Vallar, L., Grunewald, K., Feichtinger, H., Duh, Q.-Y., Clark, O.H., Kawasaki, E., Bourne, H.R. & McCormick, F. (1990). *Science **249**, 655.*

MacLeod, R.M. (1976). *Front. Neuroendocrinol. **4**, 169.*

MacLeod, R.M. & Lehmeyer, J.E. (1974). *Endocrinology **94**, 1077.*

Maddock, W.O. & Nelson, W.O. (1952). *J. Clin. Endocrinol. Metab. **12**, 985.*

Maddocks, S. & Setchell, B.P. (1989)a. *J. Endocrinol. **121**, 311.*

Maddocks, S. & Setchell, B.P. (1989)b. *Endocrinology **121**, 303.*

Maddocks, S. & Sharpe, R.M. (1989)a. *J. Endocrinol. **120**, 215.*

Maddocks, S. & Sharp, R.M. (1989)b. *J. Endocrinol. **122**, 323.*

Mahou, F.B., Gosset, F., Trinity, R.C., Madsen, P.O. (1973). *Cancer **31**, 1208.*

Majercik, M.H. & Muett, D. (1991). *Mol. Cell. Endocrinol. **75**, 247.*

Majerus, P.W., Wilson, D.B., Connolly, T.M., Bross, T.E. & Neufeld, E.J. (1985). *TIBS **10**, 168.*

Mann, D.R., Free, C., Nelson, C., Scott, C. & Collins, D.C. (1987). *Endocrinology **120**, 1542.*

Mann, D.R., Adams, S.R., Gould, K.G., Orr, T.E. & Collins, D.C. (1989). *J. Reprod. Fert. **85**, 89.*

Mann, V.Z., Newton, C.J. & Tail, G.H. (1991). *J. Mol. Endocrinol. **7**, 45.*

Mango, D., Scirpa, P., Liberati, M., Fabiano, A. & Mancuso, S. (1988). *J. Endocrinol. Invest. **11**, 521.*

Margioris, A.N., Liotta, A., Vaudry, H., Bardin, C.W. & Krieger, D.T. (1983). *Endocrinology* ***113, 663.***

Marko, M. (1984). *Eur. J. Pharmacol.* ***101, 263.***

Markstein, R. (1983). *Eur. J. Pharmacol.* ***95, 101.***

Marshall, C.J. (1987). *Cell* ***49, 723.***

Marshall, J.C., Kelch, R.P. (1986). *N. Eng. J. Med.* ***315, 1459.***

Mason, I.J., MacDonald, A.A., Laptook, A. (1984). *Biochem. Biophy. Acta.* ***795,*** *504.*

Matsumoto, A.M. (1988). *In: Textbook of medicine, pp 1404-1421. Eds J.B. Wijngaarden & L.H. Smith Jr. Philadelphia: W.B. Saunders.*

Matthews, N. (1981). *Br. J. Cancer* ***44, 418.***

Maurer, R.A. (1980)a. *J. Biol. Chem.* ***255, 8092.***

Maurer, R.A. (1980)b. *Biochemistry* ***19, 3573.***

Mayerhofer, A., Bartke, A., Amador, A.G. & Began, T. (1989). *Endocrinology* ***125, 560.***

McChesney, R., Scalfon, S.C., Tsutsumi, M., Dong, K.W., Roberts, J.L. & Bancroft, C. (1991). *Mol. Cell. Endocrinol.* ***79, R1.***

McFarland, K.C., Sprengel, R., Phillips, H.S., Köhler, M., Rosemblit, N., Nikolics, K., Segaloff, D.L. & Seeburg, P.H. (1989). *Science* ***245, 494.***

McIntoch, R.P., McIntoch, J.E.A. (1985). *Endocrinology* ***117, 169.***

McKenna, T., Lorber, D., Lacroix, A. & Rabin, D. (1979). *Acta Endocrinol.* ***91,*** *501.*

McNatty, K.P., Baird, D.T., Bolton, A., Chambers, P., Corker, C.S., MacLean, H. (1976). *J. Endocrinol.* ***71,*** *77-85.*

McNeilly, J.R., Brown, P., Clark, A.J. & McNeilly, A.S. (1991). *J. Mol. Endocrinol.* ***7, 35.***

McPhail, I.C. (1984). *Science* ***244, 622.***

Means, A.R. (1981). *Rec. Prog. Horm. Res.* ***37, 333.***

Meikle, A.W., Liu, X.-H. & Stringham, J.D. (1991). *J. Androl.* ***12, 148.***

Melis, G.B., Cagnacci, A., Gambacciani, M., Paoletti, A.M., Moggi, L. & Fioretti, P. (1988). *J. Clin. Endocrinol. Metab.* ***66, 964.***

Melis, G.B., Gambacciani, M. & Paoletti, A.M. (1987). *J. Clin. Endocrinol. Metab.* ***65, 541.***

Melner, M.H. & Abney, T.O. (1980). *J. Steroid Biochem.* ***13, 203.***

Mendelson, C.R., Cleland, W.H., Smith, M.E. & Simpson, E.R. (1982). *Endocrinology* ***111, 1077.***

Mendelson, C.R., Dufau, M. & Catt, K. (1975). *J. Biol. Chem.* ***250, 8818.***

Mendelson, C.R., Corbin, C.J., Smith, M.E., Smith, J., Simpson, E.R. (1986). *Endocrinology* ***118, 968.***

Mendelson, C.R., Evans, C.T. & Simpson, E.R. (1987). *J. Steroid Biochem.* ***27,*** *753.*

Mendelson, C.R. & Simpson, E.R. (1987). *Mol. Cell. Endocrinol.* ***52, 169.***

Mendelson, C.R., Means, G.D., Mahendroo, M.S., Corbin, C.J., Steinkampf, M.P., Graham-Lorence, S. & Simpson, E.R. (1990). *Biol. Reprod.* ***42, 1.***

Mendis-Handagama, S.M.L.C., Risbridger, G.P. & de Kretzer, D.M. (1987). *Int. J. Androl.* ***10, 525.***

Mendis-Handagama, S.M.L.C., Watkins, P.A., Gelber, S.J., Scallen, T.J., Zirkin, B.R. & Ewing, L.L. (1990). *Endocrinology* ***127, 2947.***

Menon, K.M.J., Dorfman, R.I. & Forchielli, E. (1967). *Biochim. Biophys. Acta* ***148, 486.***

Merchenthaler, I., Culler, M.D., Petrusz, P. & Negro-Vilar, A. (1987). *Mol. Cell. Endocrinol.* ***54, 239.***

Meunier, H., Rivier, C., Evans, R.M. & Vale, W. (1988). *Proc. Natl. Acad. Sci. USA* ***85, 247.***

Meyer, A.S. (1955). *Biochim. Biophys. Acta* ***17, 441.***

Michell, R.H. (1975). *Biochim. Biophys. Acta* ***415, 81.***

Michell, R.H. (1979). *TIBS* ***4, 128.***

Miell. J.P., Pralong, F.P., Coeder, R. & Gaillard, R.C. (1990). *Clin. Endocrinol. Metab.* ***71, 1519.***

Miller, S. C., Bowman, B. M. & Rowland, H. G. (1983). *Am. J. Anat.* ***168, 1.***

Miller, W.L. (1988). *In: Endocrine genes analytical methods experimental approaches and selected systems, pp 119-135. Eds Yun-Fai Lau. Oxford University Press: New York.*

Miller, W.R. & O'Neill, J. (1987). *Steroids* ***50, 537.***

Miller, W.R. (1989). *Cancer Treat. Rev.* ***16, 83.***

Minegishi, T., Kusuda, S. & Dufau, M.L. (1987). *J. Biol. Chem.* ***262, 17138.***

Mineur, P., de Cooman, S., Hustin, J., Verhoeven, G. & de Hertogh, R. (1987). *J. Clin. Endocrinol. Metab.* ***64, 686.***

Missale, C., Memo. M., Liberini, P., Carruba, M.O. & Spano, P. (1985). *Eur. J. Pharm.* ***109, 315.***

Moger, W.H. (1979). *J. Endocrinol.* ***80, 321.***

Moger, W.H. (1980). *J. Steroid Biochem.* ***13, 61.***

Moger, W.H. (1985). *Life Sciences* ***37, 869.***

Moger, W.H. (1991). *Endocrinology* ***128, 1414.***

Molcho, J., Zakut, H. & Naor, Z. (1984)a. *Endocrinology* ***114, 1048.***

Molcho, J., Ell, Y., Zakut, H. & Naor, Z. (1984)b. *Endocrinology* ***114, 2382.***

Molenaar, R., Rommerts, F.F.G. & van der Molen, H.J. (1983). *Int. J. Androl.* ***6, 261.***

Molenaar, R., Rommerts, F.F.G. & van der Molen, H.J. (1984). *In: Recent progress in cellular endocrinology of the testis Vol. 123, pp 257-264.*

Molenaar, R., de Rooij, D.G., Rommerts, F.F.G. & van der Molen, H.J. (1986). *Endocrinology* ***118, 2546.***

Mollner, S. & Pfeuffer, T. (1988). *Eur. J. Biochem.* ***171, 265.***

Monder, C. & Lakshmi, V. (1990). *Endocrinology* ***126, 2435.***

Monsma, F.G., McVittie, L.D., Gerfen, C.R., Mahan, L.C. & Sibley, D.R. (1989). *Nature **342, 926.***

Morales, C., Hugly, S. & Gliswold, M.D. (1987). *Biol. Reprod. **36, 1035.***

Morato, T., Hayano, M., Dorfman, R.I. & Axelrod, L.R. (1961). *Biochim. Biophys. Res. Commun. **6, 334.***

Mordes, J.P., Longcope, C., Flatt, J.P., MacLean, D.B., Rossini, A.A. (1984). *Endocrinology **115, 167.***

Mori, H., christensen, A.K. (1980). *J. Cell Biol. **84, 340.***

Mori, H., Hiromoto, N., Nakahara, M. & Shiraishi, T. (1982). *J. Clinical Endocrinol. Metab. **55, 634.***

Morii, S., Naka, Y., Inui, T. & Shintani, H. (1988). *Cancer Res. **48, 4395.***

Morra, M., Leboulenger, F., Desrues, L., Tonon, M.-C. & Vaudry, H. (1991). *Endocrinology **128, 2625.***

Morra, M., Leboulenger, F., Homo-Delarche, F., Netchtaflo, P. & Vaudry, H. (1989). *Life Science **45, 175.***

Morra, M., Leboulenger, F. & Vaudry, H. (1990). *Endocrinology **127, 218.***

Morre, R.J. & Wilson, J.D. (1972). J. Biol. Chem. **247**, 958.

Morris, P.L. & Saxena, B.B. (1980). *Endocrinology **107, 1639.***

Morris, I.D., Lendon, R.G. & Zaidi, A. (1988). *J. Endocrinol. **119, 467.***

Moses, H.L., Yang, E.Y. & Pietenpol, J.A. (1990). *Cell **63, 245.***

Mostofi, T.K. & Bresler, V.M. (1976). *In: Pathology of tumours in laboratory animals, vol.I, tumours of the rat, part 2, pp135-150. V.S. Turusov (ed). Lyon, France, World Health Organization International Agency for Research on Cancer.*

Mostofi, F.K. & Bresler, V.M. (1979). *In: Pathology of tumours in laboratory animals: tumors of the mouse, Vol 2, pp 325-348. Ed V.S. Turusov. IARC Scientific Publications No 23, International Agency for Research on Cancer, Lyon.*

Mostofi, E.K. & Price, E.B. (1973). *Atlas of tumour pathology. Second series, fascile 8. AFIP, pp 86-99.*

Moyle, W.R. (1975). *J. Biol. Chem.* ***250, 9163.***

Moyle, W.R. & Ramachandran, J. (1973). *Endocrinology* ***93, 127.***

Mueller, G.P., Simpkins, J., Meites, J., Moore, K.E. (1976). *Neudocrinology* ***20, 121.***

Mukhopadhyay, A.K. & Schumacher, M. (1985). *FEBS Lett* ***187, 56.***

Muller, R. (1986). *TIBS* ***11, 129.***

Muller, R., Slamon, D.J., Tremblay, J.M., Cline, M.J. & Verma, I.M. (1982). *Nature* ***209, 640.***

Munemura, M., Cote, T.E., Tsuruta, K., Eskay, R.L. & Kebabian, J.W. (1980). *Endocrinology* ***107, 1676.***

Munemura, M., Agui, T. & Sibley, D.R. (1989). *Endocrinology* ***124, 346.***

Murono, E.P. & Payne, A.H. (1976). *Biochim. Biophys. Acta* ***450, 89.***

Murono, E.P. & Payne, A.H. (1979). *Biol. Reprod.* ***20, 911.***

Nagy, L. & Freeman, D.A. (1990). *Endocrinology* ***126, 2267.***

Nakajin, S. & Hall, P.F. (1981). *J. Biol. Chem.* ***256, 3871.***

Nakajin, S., Shively, J.E., Yuan, P.M. & Hall, P.F. (1981). *Biochemistry* ***20, 4037.***

Nakamura, T. & Ui, M. (1985). *J. Biol. Chem.* ***260, 3584.***

Nakhla, A.M., Mather, J.P., Jänne, O.A. & Bardin, C.W. (1984). *Endocrinology* ***115, 121.***

Nangalama, A.W. & Moberg, G.P. (1991). *J. Endocrinol.* ***131, 87.***

Naor, Z. (1990). *Endocrinology* ***126, 1521.***

Naville, D., Chatelain, P.G., Avallet, O. & Saez, J.M. (1990). *Mol. Cell. Endocrinol.* ***70, 217.***

Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R. & Lefkowith, J.B. (1986). *Ann. Rev. Biochem.* ***55, 69.***

Negro-Vilar, A., Ojeda, S.R. & McCann, S.M. (1979). *Endocrinology* ***104, 1749.***

Neher, R. & Wettstein, A. (1960). *Acta Endocrinol.* ***35, 1.***

Nestler, J.E. (1989). *Endocrinology* ***125, 2127.***

Nestler, J.E. (1990). *Endocrinology* ***127, 2064.***

Nikula, H. & Huhtaniemi, I. (1988). *Mol. Cell. Endocrinol.* ***55, 53.***

Nielsen, J.R., Hansen, H.S. & Jensen, B. (1989). *Mol. Cell. Endocrinol.* ***61, 181.***

Nimrod, A., Ryan, K.J. (1975). *J. Clin. Endocrinol. Metab.* ***40, 367-372.***

Nishihara, M., Winters, C.A., Buczko, E., Waterman, M.R., Dufau, M.L. (1988). *Biochim. Biophys. Res. Commun.* ***154, 151.***

Nishizuka, Y. (1984). *Nature* ***308, 693.***

Nishizuka, Y. (1986). *Science* ***233, 305.***

Niznik, H.B. (1987). *Mol. Cell. Endocrinol.* ***54, 1-22.***

Noguchi, S., Nishizawa, Y., Takatsuka, D., Sato, B. & Matsumoto, K. (1986). *Cancer Res.* ***46, 573.***

Northup, J.K., Renart, M.F., Grove, J.R. & Mansour, T.E. (1980). *J. Biol. Chem.* ***10, 11861.***

Norton, J.N. & Skinner, M.K. (1989). *Endocrinology* ***124, 2711.***

Nowell, P.C. & Croce, C.M. (1988). *FASEB J.* ***2, 3054.***

Novak, E.R. & Long, J.H. (1965). *Am. J. Obstet. Gynecol.* ***92, 1082.***

Nozu, K., Dehejia, A., Zawistowich, L., Catt, K.J. & Dufau, M.L. (1981)b. *J. Biol. Chem.* ***256, 12875.***

Nozu, K., Dufau, M.L. & Catt, K.J. (1981)a. *J. Biol. Chem.* ***256, 1915.***

Nozu, K., Dufau, M.L. & Catt, K.J. (1981)c. *J. Biol. Chem.* ***256, 1915.***

Nozu, K., Matsuura, S., Catt, K.J. & Dufau, M.L. (1981)d. *J. Biol. Chem.* ***256, 10012.***

Ohashi, M., Hasegawa, Y., Haji, M., Igarashi, M. & Nawata, H. (1990). *Clin. Endocrinol.* ***33, 613.***

Ohata, M. (1979). *Archivum Histogicum Japoonicum* ***42, 51.***

Ohta, H., Okajima, F. & Ui, M. (1985). *J. B3iol. Chem.* ***260, 15771.***

Ojeifo, J.O., Byers, S.W., Papadopoulos, V. & Dym, M. (1990). *J. Reprod. Fert.* ***90, 93.***

O'Leary, P.C., Jackson, A.E. & de Kretzer, DD.M. (1986). *Mol. Cell. Endocrinol.* ***48, 51.***

O'Neill, J., Elton, R.A. & Miller, W.R. (19800). *Br. Med. J.* ***296, 741.***

O'Neill, J.S. & Miller, W.R. (1987). *Br. J. CDancer* ***56, 601.***

Onoda, M. & Djakiew, D. (1990). *Mol. Cell. . Endocrinol.* ***73, 35.***

Onoda, M., Djakiew, D. & Papadopoulos, V. ((1991). *Mol. Cell. Endocrinol.* ***77,*** *207.*

Oonk, R.B., Krasnow, J.S., Beattie, W.C. && Richards, J.S. (1989). *J. Biol. Chem.* ***264,*** *21934.*

Orava, M., Voutilainen, R., Vihko, R. (1989).. *Mol. Endocrinol.* ***3,*** *887.*

Orczyk, G.P., Mordes, J.P. & Longcope, C. ((1987). *Endocrinology* ***120,*** *1482.*

Orth, J. & Christensen (1977). *Endocrinology* ***101,*** *262.*

Ortlip, S.A., Li, S.A. & Li, J.J. (1981). *Endoocrinology* ***109,*** *1331.*

Osawa, Y., Higashiyama, T., Fronckowiak, M1., Yoshida, N. & Yarborough, C. (1987). *J. Steroid Biochem.* ***27,*** *781.*

O'Shaughnessy P. J., Wong K-. L. (1981). *Endocrinology* ***109,*** *1061.*

O'Shaughnessy, P.J. & Payne, A.H. (1982). *J.. Biol. Chem.* ***257,*** *11503.*

Padmanabhan, V., Keech, C. & Convey, E.M.. (1983). *Endocrinology* ***112,*** *1782.*

Page, R.B. & Bergland, R.M. (1977). *Am. J. Anat.* ***148,*** *345.*

Pakarinen, P. & Huhtaniemi, I. (1989). *J Moll. Endocrinol.* ***3,*** *139.*

Pakarinen, P., Vihko, K.K., Voutilainen, R. & Huhtaniemi, I. (1990). *Endocrinology* ***127,*** *2469.*

Palmer, J.M. (1991). *In: Puberty and its disorders, endocrinology and metabolism, clinics of north America, Vol 20, pp 231-240.*

Pandey, K.N. & Inagami, T. (1986). J. Biol. Chem. **261**, 3934.

Pandiella, A., Beguinot, L., Vicentini, L.M. & Meldolesi, J. (1989). *TIPS **10**, 411.*

Papadopoulos, V., Carreau, S. & Drosdowsky, M.A. (1985). *FEBS Letters **188**, 312.*

Papadopoulos, V., Drosdowsky, M.A. & Carreau, S. (1986). *Andrologia **18**, 79.*

Papadopoulos, V., Kamtchouing, P., Drosdowsky, M.A., Hochereau de Reviers, M.T. & Carreau, S. (1987). *J. Endocrinol. **114**, 459.*

Papadopoulos, V., Brown, A.S. & Hall, P.F. (1990)a. *Mol. Cell. Endocrinol. **74**, 109.*

Papadopoulos, V., Mukhin, A.G., Costa, E. & Krueger, K.E. (1990)b. *J. Biol. Chem. **266**, 3772.*

Papavasiliou, S.S., Zmeili, S., Khoury, S., Landefeld, T.D., Chin, W.W., Marshall, J.C. (1986). *Proc. Natl. Acad. Sci. USA **83**, 4026.*

Park, Y., Park, S.D., Cho, W.K., Kim, K. (1988). *Brain Res. **451**,, 255.*

Parker, J., Daniel, L.W. & Waite, M. (1987). *J. Biol. Chem. **262**, 5385.*

Parker, S.L., Kalra, S.P. & Crowley, W.R. (1991). *Endocrinology **128**, 2309.*
Parmentier, M., Inagami, T., Pochet, R., Desclin, J.C. (1983). *Endocrinology **112**, 1318.*

Parmentier, M., Libart, F., Maenhaut, C., Lefort, A., Gerard, C., Perret, J., Vansande, J., Dumont, J.E., Vassart, G. (1989). *Science **246**, 1620.*

Parvinen, M., Nikula, H. & Huhtaniemi, I. (1984). *Mol. Cell. Endocrinol. **37**, 331.*

Pasanen, M. (1985). *Biol. Res. Pregnancy **6**, 94.*

Pasqualini, J.R., Gelly, C., Nguyenm, B. -L. & Vella, C. (1989). *J. Steroid Biochem **34**, 155.*

Pattilo, R.A., Hussa, R.O., Huang, W.Y., Delfts, E., Mattingly, R.F. (1972). *J. Clin. Endocrinol. Metab. **34**, 59.*

Payne, A.H. (1990). *Biol. Reprod.* ***42, 399.***

Payne, A. H. Downing, J. R. & Wong, K. -L. (1980)a. *Endocrinology* ***106, 1424.***

Payne, A.H., Kelch, R.P., Musich, S.S. & Halpern, M.E. (1976). Intratesticular site of aromatization in the human. *J. Clin. Endocrinol. Metab.* ***42, 1081.***

Payne, A.H., Quin, P.G. & Stalvey, J.R.D. (1985). The stimulation of steroid biosynthesis by luteinizing hormone. *In: Luteinizing hormone action and receptors, pp 135-172, M. Ascoli (ed). CRC Press: Boca Raton.*

Payne, A.H., Wong, K.-L. & Vega, M.M. (1980)b. *J. Biol. Chem.* ***255, 7118.***

Payne, A.H., O'Shaughnessy, P.J., Chase, D.J., Dixon, G.E.K. & Christensen, A.K. (1982). *Ann. N.Y. Acad Sci.* ***383, 174.***

Paz, G.F., Winter, J.S.D., Reyes, F.I. & Faiman, C. (1980). *Steroids* ***36, 661.***

Paz, G.F., Winter, J.S.D., Reyes, F.I. & Faiman, C. (1980). *Steroids* ***36, 675.***

Paz, G.F., Winter, J.S.D., Reyes, F.I. & Faiman, C. (1982). *In. J. Androl.* ***5,*** *74.*

Pederson, R.C. & Brownie, A.C. (1987). *Science* ***236, 188.***

Pellegrini, I., Gunz, G., Grisoli, F. & Jaquet, P. (1990). *Endocrinology* ***126,*** *1087.*

Pelosin, J.-M., Ricouart, A., Sergheraert, C., Benahmed, M. & Chambaz, E.M. (1991). *Mol. Cell. Endocrinol.* ***75, 149.***

Perel, E., Stolee, K.H., Kharlip, L., Blackstein, M.E. & Killinger, D.W. (1984). *J. Clin. Endocrinol. Metab.* ***58, 467.***

Perel, E., Daniilescu, D., Kharlip, L., Blackstein, M. & Killinger, D.W. (1988). *J. Steroid Biochem.* ***29, 393.***

Pereira, M.E., Segaloff, D.L., Ascoli, M. (1988)a. *Endocrinology* ***122, 2232.***

Pereira, M.E. Segaloff, D.L., Ascoli, M. (1988)b. *J. Biol. Chem.* ***263, 9761.***

Pereira, M.E., Segaloff, D.L., Ascoli, M. & Eckstein, F. (1987). *J. Biol. Chem.* ***262, 6093.***

Perkins, L.M. & Payne, A.H. (1988). *Endocrinology* ***123, 2675.***

Perlman, P.L. (1950). *Endocrinology **46**, 341.*

Perrard-Sapori, M.H., Chatelain, P., Vallier, P. & Saez, J.M. (1986). *Biochim. Biophys. Res. Commun. **134**, 957.*

Persson, H., Ayer-Lievre, C., Soder, O., Villar, M.J., Metsis, M., Olson, L., Ritzen, M. & Hokfelt, T. (1990). *Science **247**, 704.*

Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H. & Billard, R. (1986). *Rec. Prog. Horm. Res. **42**, 513.*

Pfeifer-Ohlsson, S., Goustin, A.S., Rydnet, J., Wahlstroem, R., Bjersing, L., Atehelmn D. & Ohlsson, R. (1984). *Cell **35**, 585.*

Pfeiffer, D.G., Pfeiffer, A., Almeida, O.F.X. & Herz, A. (1987). *J. Endocrinol. **114**, 469.*

Pierce, J.G. & Parsons, T.F. (1981). *Ann. Rev. Biochem. **50**, 465.*

Pierrepoint, C.G., Griffiths, K., Grant, J.K. & Stewart, J.S.S. (1966). *J. Endocrinol. **35**, 409.*

Phillips, D.M., Lakshmi, V. & Monder, C. (1989). *Endocrinology **125**, 209-216.*

Pignataro, O.P., Radicella, J.P., Calvo, J.C. & Charreau, E.H. (1983). *Mol. Cell. Endocrinol. **33**, 53.*

Pineau, C., Sharpe, R.M., Saunders, P.T.K., Gérard, N. & Jégou, B. (1990). *Mol. Cell. Endocrinol. **72**, 13.*

Pirke, K.M., Krings, B. & Vogt, H.-J. (1979). *Acta Endocrinol. **92**, 358.*

Piquette, G.N., Kenney, R.M., Sertich, P.L., Yamoto, M. & Hsueh, A.J.W. (1990). *Biol. Reprod. **43**, 1050.*

Platts, E.A., Schulster, D. & Cooke, B.A. (1988). *Biochem. J. **253**, 895.*

Plotsky, P.M., Cunningham, E.T. & Widmaier, E.P. (1989). *Endocr. Rev. **10**, 437.*

Podesta, E.J., Dufau, M.L., Solano, A.R. & Catt, K.J. (1978). *J. Biol. Chem. **253**, 8994.*

Pokel, J.D., Moyle, W.R. & Greep, R.O. (1972). *Endocrinology **91**, 323.*

Pöllänen, P. & Maddocks, S. (1988). *J. Reprod. Fert. **82**, 437.*

Pomerantz, D.K. (1981). *Endocrinology **109, 2004.***

Privalle, C.T., Crivello, J.F. & Jefcoate, C.R. (1983). *Proc. Natl. Acad. Sci. USA **80, 702.***

Privalle, C.T., McNamara, B.C., Dhariwal, M.S. & Jefecoate, C.R. (1987). *Mol. Cell. Endocrinol. **53, 87.***

Puente, M. & Catt, K. (1986). *J. Steroid Biochem. **25, 917.***

Purvis, J.L., Canick, J.A., Latif, S.A., Rosenbaum, J.H., Hollogitas, J., Menard, R.H. (1973)a. *Arch. Biochem. Biophy. **159, 39.***

Purvis, J.L., Canick, J.A., Rosenbaum, J.H., Hollogital, J., Latif, S.A. (1973)b. *Arch. Biochem. Biophy. **159, 32-38.***

Purvis, Cusan & Hansson (1981)a. *J. Biol. Chem. **15, 77.***

Purvis, K. & Hansson, V. (1978). *Mol. Cell. Endocrinol. **12, 123.***

Purvis, K., Olsen, A. & Hansson, V. (1981)b. *J. Biol. Chem. **256, 11434.***

Quigley, M.E., Sheehan, K.L., Casper, R.F., Yen, S.S.C. (1980). *J. Clin. Endocrinol. Metab. **50, 949.***

Quinn, P.G., Dombrausky, L.J., Chen, Y.-D. & Payne, A.H. (1981). *Endocrinology **109, 1790.***

Quinn, P.G., Georgiou, M. & Payne, A.H. (1985). *Endocrinology **116, 2300.***

Quinn, P.G. & Payne, A.H. (1984). *J. Biol. Chem. **259, 4130.***

Quinn, P.G. & Payne, A.H. (1985). *Endocrinology **116, 2300.***

Raburn, D.J., Coquelin, A. & Hitson, J.C. (1991). *Biol. Reprod. **45, 172.***

Raeside, J.I., Berthelon, M.C., Sanchey, P., Saez, J.M. (1988). *Biochim. Biophys. Res. Commun. **151, 163.***

Rainey, W.E., Viard, I., Mason, J.I., Cochet, C., Chambaz, E.M. & Saez, J.M. (1988). *Mol. Cell. Endocrinol. **60, 189.***

Ramaley, J.A. (1979). *Biol. Reprod. **20, 1.***

Rani, C.S.S & Payne, A.H. (1986). *Endocrinology **118, 1222.***

Ranta, T., Chen, H.-C., Jalkanen, J., Nikula, H., Shimohigashi, Y. & Huhtaniemi, I. (1987). *Obst. Gynecol.* ***70, 171.***

Rasmussen, D.D., Liu, J.H., Wolf, P.L. & Yen, S.S.C. (1986). *J. Clin. Endocrinol. Metab.* ***62, 479.***

Rasmussen, H. (1989). *Scientific American* ***260, 44.***

Rasmussen, H. & Barrett, P.Q. (1984). *Physiol. Rev.* ***64, 938.***

Raymond, V., Bealieu, M., Labrie, F., Boissier, J.R. (1978). *Science* ***200, 1173.***

Reame, N., Sauder, S.E., Kelch, R.P., Marchall, J.C. (1984). *J. Clin. Endocrinol. Metab.* ***59, 328.***

Rebois, R.V. & Patel, J. (1985). *J. Biol. Chem.* ***260, 8026.***

Redding, T.W., Coy, D.H. & Schally, A.V. (1982). *Pro. Natl. Acad. Sci.* ***79, 1273.***

Reddy, J., Svoboda, D. (1972)a. *J. Histochem. Cytochem.* ***20, 140.***

Reddy, J., Svoboda, D. (1972)a. *J. Histochem. Cytochem.* ***20, 793.***

Reddy, J.K., Rao, M.S., Azarnoff, D.L. & Sell, S. (1979). *Cancer Res.* ***39, 152.***

Reed, M.J. (1991). *J. Endocrinol.* ***129, 163.***

Reiko, T. & Werb, Z. (1984). *Am. J. Physiol.* ***246, C1.***

Rempel, S.A. & Johnston, R.N. (1988). *Development* ***104, 87.***

Reure, S., Maenhaut, C. & Dumont, J.E. (1990). *Exp. Cell Res.* ***189, 33.-40.***

Rich, K.A & de Kretzer, D.M. (1977). *Endocrinology* ***101, 959.***

Riegle, G.D. & Meites, J. (1976). *Proc. Soc. Exp. Biol. Med.* ***151, 507.***

Ringston, S.J. & Schwartz, N.B. (1985). *Endocrinology* ***116, 472.***

Risbridger, G.P., Kerr, J.B. & de Kretzer, D.M. (1981)a. *Biol. Reprod.* ***24, 534.***

Risbridger, G.P., Kerr, J.B., Peake, R.A. & de Kretzer, D.M. (1981)b. *Endocrinology* ***109, 1234.***

Risbridger, G.P., Kerr, J.B., Peake, R., Rich, K.A. & de Kretzer, D.M. (1981)c. *J. Reprod. Fert.* ***63, 415.***

Risbridger, G. P. & de Kretser, D. M. (1986). *J. Reprod. Fert.* ***76, 331-338.***

Risbridger, G.P., Drummond, A.E., Kerr, J.B. & de Kretzer, D.M. (1987). *J. Reprod. Fert.* ***81, 617.***

Risbridger, G.P., Clements, J., Robertson, D.M., Drummond, A.E., Muir, J., Burger, H.G. & de Kretser, D.M. (1989). *Mol. Cell. Endocrinol.* ***66, 119.***

Ritta, M.N., Campos, M.B. & Calandra, R.S. (1987). *Life Science* ***40, 791.***

Rivarola, M.A. & Podesta, E.J. (1972). *Endocrinology* ***90, 618.***

Rivarola, M.A., Podesta, E.J. & Chemes, H.E. (1972). *Endocrinology* ***91, 537.***

Rivarola, M.A., Sanchey, P. & Saez, J.M. (1985). *Endocrinology* ***117, 1796.***

Roberts, K.P. & Griswold, M.D. (1989). *Endocrinology* ***125, 1174.***

Roberts, S.A., Nett, T.M., Hartman, H.A, Adams, T.E. & Stoll, R.E. (1989). *J. Am. Coll. Toxicol.* ***8, 487.***

Robertson, D.M., Foulds, L.M., Lversha, L., Morgan, F.J., Hearn, M.T.W., Burger, H.G., Wettenhall, R.E.H. & de Kretser, D.M. (1985). *Biochim. Biophys. Res. Commun.* ***126, 220.***

Rodbel, M. (1985). *TIBS* ***10, 461.***

Rommerts, F.F.G. & Cooke, B.A. (1988). *In: Hormones and their actions, Part II, pp 163-179. Elsevier.*

Rommerts, F.F.G., Cooke, B.A., van der Kemp, J.E.C.M. & van der Molen, H.J. (1972). *FEBS Letters* ***24, 251.***

Rommerts, F.F.G., Grootenhuis, A.J., Hoogerbrugge, J.W. & van der Molen, H.J. (1985). *Mol. Cell. Endocrinol.* ***42, 105.***

Ronco, A.M., Pino, A.M. & Valladares, L.E. (1988). *J. Endocrinol.* ***116, 387.***

Ropert, J.F., Quigley, M.E., Yen, S.S.C. (1984). *Life Science* ***34, 2067.***

Roosen-Runge, E.C. & Anderson, D. (1959). *Acta Anat.* ***37, 125.***

Roselli, C.E., Horton, L.E., Resko, J.A. (1985). *Endocrinology* ***117, 2471.***

Roselli, C.E., Kelly, M.J. & Ronnekleiv, O.K. (1990). *Endocrinology **126, 1080.***

Rosen, H., Dalkin, A., Haisenleder,D., DeMott Friberg, R., Ortoland, G. & Barkan, A. (1991). *Endocrinology **128, 654.***

Rosengurt, E. (1986). *Science **234, 161.***

Rotszstein, W.H., Charli, J.L., Pattou, E., Epelbaum, J., Kordon, C. (1976). *Endocrinology **99, 1663.***

Rouiller, V., Gangnerau, M.N., Vayssiere, J.L. & Picon, R. (1990). *Mol. Cell. Endocrinol. **72, 111.***

Rubens, R., Dhont, M. & Vermeulen, A. (1974). *J. Clin. Endocrinol. Metab. **39, 40.***

Rudman, D., Kutner, M.H., Rogers, C.M., Lubin, M.F., Fleming, A., Bain, R.P. (1981). *J. Clin. Invest. **67, 1361.***

Russell, L.D., Amlani, S.R., Vogl, A.W. & Weber, J.E. (1987). *Am. J. Anat. **178, 231.***

Ryan, R.J., Charlesworth, M.C., McCormick, D.J., Milius, R.P. & Keutmann, H.T. (1988). *FASEB J. **2, 2661.***

Saade, G., London, D.R. & Clayton, R.N. (1989)a. *Endocrinology **124, 1744.***

Saade, G., London, D.R., Lalloz, M.R.A. & Clayton, R.N. (1989)b. *J. Mol. Endocrinol. **2, 213.***

Saez, J.M., Morera, A.-M. & Haour, F. (1979). *Horm. Cell Reg. **3, 187.***

Saez, J.M., Morera, A.M., Haour, F. & Evain, D. (1977). *Endocrinology **101, 1256.***

Sagi-Eisenberg, R. (1989). *TIBS **14, 355.***

Sahu, A., Kalra, P.S., Crowley, W.R. & Kalra, S.P. (1990). *Endocrinology **127, 2307.***

Sairam, M.R. (1990). *Biochem. J. **265, 667.***

Sakakura, M., Takebe, K. & Nakagawa, S. (1975). *J. Clin. Endocrinol. Metab. **40, 774.***

Salesse, R., Genry, N. & Garnier, J. (1983). *Biol. Cell. **49, 187.***

Samuels, L.T., Bussman, L., Malsumoto, K. & Houseby, R.A. (1975). *J. Steroid Biochem. 6, 291.*

Samuels, L.T., Uchikawa, T., Zain-ul-Abedin, M. & Huseby, R.A. (1969). *Endocrinology 85, 96.*

Sar, M., Lubahn, D.B., French, F.S. & Wilson, E.M. (1990). *Endocrinology 127, 3180.*

Sar, M., Sahu, A., Crowley, W.R. & Kalra, S.P. (1990). *Endocrinology 127, 2752.*

Sarkar, D.R. & Yen, S.S.C. (1985). *Endocrinology 116, 2080.*

Sato, B., Huseby, R.A. & Samuels, L.T. (1978). *Cancer Res. 38, 2842.*

Sato, B., Maeda, Y., Nakao, Noma, K., Kishimoto, S. & Matsumoto, K. (1985). *Eur. J. Cancer Clin. Oncol. 21, 199.*

Scallen, T.J. & Sanghvi, A. (1983). *Proc. Natl Acad. Sci. 80, 2477.*

Schaison, G., Durand, F. & Mowszowicz, I. (1978). *Acta Endocrinol 89, 126.*

Schlegel, W., Kempner, E.S. & Rodbell, M. (1979). J. Biol. Chem. **254**, 5168.

Schmidt-Gollwitzer, M., Eiletz, J., Genz, T. & Pollow, K. (1979). *J. Clin. Endocrinol. Metab. 49, 212.*

Schneider, H.P.G. & McCann, S.M. (1969). *Endocrinology 85, 121.*

Schoen, E.J. (1964). *Endocrinology 75, 56.*

Schumacher, M., Schafer, G., Holstein, A. F. & Hilz, H. (1978). *FEBS Letters 91, 333.*

Scott, I.S., Charlton, H.M., Cox, B.S., Grocock, C.A., Sheffield, J.W. & O'Shaughbessy, P.J. (1990). *J. Endocrinol. 125, 131.*

Seamon, K.B. & Daly, J.W. (1981). *J. Biol. Chem. 256, 9799.*

Seamon, K.B., Vaillancourt, R., Edwards, M. & Daly, J.W. (1984). *Proc. Natl. Acad. Sci. USA 81, 5081.*

Seemon, P., Lee, T., Chau-Wong, M. & Wong, K. (1976). *Nature 261, 717.*

Seemon, P. & Niznik, H.B. (1990). *FASEB J. 4, 2737.*

Seemon, P., Niznik, H.B., Guan, H.-C., Booth, G. & Ulpian, C. (1989). *Proc. Natl Acad. Sci. USA* ***86, 10156.***

Segaloff, D.L., Ascoli, M. & Puett, D. (1981). *Biochim. Biophy. Acta* ***675, 351.***

Segaloff, D.L., Sprengel, R., Nikolics, K. & Ascoli, M. (1990). *Rec. Prog. Horm. Res.* ***46, 261.***

Selmanoff, M., Shu, C., Petersen, S.L., Barraclough, C.A. & Zoeller, R.T. (1991). *Endocrinology* ***128, 459.***

Selye, H. (1939). *Endocrinology* ***25, 615.***

Senogles, S.E., Benovic, J.L., Amlaiky, N., Unson, C., Milligan, G., Vinitsky, R., Spiegel, A.M. & Caron, M.G. (1987). *J. Biol. Chem.* ***262, 4860.***

Seppelt, U. (1978). *J. Clin. Endocrinol. Metab.* ***47, 1230.***

Serio, M. & Fiorelli, G. (1991). *Mol. Cell. Endocrinol.* ***78, C77.***

Serri, O., Beauregard, H., Lesage, J., Pedneault, L., Comtois, R., Jilwan, N., Somman M., Vachon, L. & Brownell, J. (1990). *J. Clin. Endocrinol. Metab.* ***71, 682.***

Setchell, B.P. (1978). *In: The mammalian testis. Elek Books London and Cornell University Press: Ithaca.*

Sethumadhavan, K., Bellino, F.L. & Thotakura, N.R. (1991). *Mol. Cell. Endocrinol.* ***78, 25.***

Shaha, C., Morris, P.L., Chen, C.-L.C., Vale, W. & Bardin, C.W. (1989). *Endocrinology* ***125, 1941.***

Shaar, C.J., Euker, J.S., Riegle, G.D., Meites, J. (1975). *J. Endocrinol.* ***66, 45.***

Sharpe, R.M. (1976). *Nature* ***264, 644.***

Sharpe, R.M. (1980). *Nature* ***286, 12.***

Sharpe, R.M. (1983). *Quart. J. Exp. Physiol.* ***68, 265.***

Sharpe, R.M. (1984). *Biol. Reprod.* ***30, 29.***

Sharpe, R.M. (1986). *Clinics Endocrinol. Metab.* ***15, 185.***

Sharpe, R.M. (1990). *Clinical Endocrinology* ***33, 787.***

Sharpe, R.M., Bartlett, J.M.S. & Allenby, G. (1991). *J. Endocrinol.* ***128, 359.***

Sharpe, R.M. & Cooper, I. (1982). *Mol. Cell. Endocrinol.* ***26, 141.***

Sharpe, R.M. & Cooper, I (1983). *J. Reprod. Fert.* ***69, 125.***

Sharpe, R.M. & Cooper, I. (1984). *Mol. Cell. Endocrinol.* ***37, 159.***

Sharpe, R.M. & Fraser, H.M. (1980). *Nature* ***287, 642.***

Sharpe, R.M. & Fraser, H.M. (1983). *Mol. Cell. Endocrinol.* ***33, 131.***

Sharpe, R.M., Fraser, H.M., Cooper, I. & Rommerts, F.F.G. (1981). *Nature* ***290, 785.***

Sharpe, R.M. & Harmer, T.J. (1983). *Horm. cell regul.* ***7, 217.***

Sharpe, R.M., Kerr, J.B. & Maddocks, S. (1988). Mol. Cell. Endocrinol. **60**, 243.

Sharpe, R.M. & McNeilly, A.S. (1979). *Mol. Cell. Endocrinol.* ***16, 19.***

Shaw, M.J., Georgopoulos, L.E. & Payne, A.H. (1979). *Endocrinology* ***104, 912.***

Sheckler, C.B., Matsumoto, A.M. & Bremner, W.J. (1989). *J. Clin. Endocrinol. Metab.* ***68, 397.***

Shikita, M. & Hall, P.F. (1967). *Biochim. Biophy. Acta* ***136, 484.***

Shikita, M. & Hall, P.F. (1973)a. *J. Biol. Chem.* ***248, 5598.***

Shikita, M. & Hall, P.F. (1973)b. *Biochem. Biophys. Acta.* ***136, 484.***

Shikita, M. & Tamaoki, B.-I. (1965). *Endocrinology* ***76, 563.***

Shimohigashi, Y. & Chen, H.-C. (1982). *FEBS Letters* ***150, 64.***

Sibley, D.R. (1991). *TIPS* ***12, 7.***

Siegel, R.A. & Prentice, D.E. (1987). *Sandoz Internal Report, Basle, Switzerland.*

Sikora, K., Evan, G., Stewart, J, Watson, J.V. (1985). *Br. J. Cancer* ***52, 171.***

Silavin, S.L., Javitt, N.B. & Strauss, III J.F. (1984). *Endocrinology* ***115, 1511.***

Simmons, D.L., Lalley, P.A. & Kasper, C.B. (1985). *J. Biol. Chem.* ***260, 515.***

Simpkins, J.W., Mueller, G.P., Huang, H.H. & Meites, J. (1977). *Endocrinology **100**, 1672.*

Simpson, B.J.B., Risbridger, G.P., Hedger, M.P. & de Kretser, D.M. (1991). *Mol. Cell. Endocrinol. **75**, 49.*

Simpson, E.R. (1979). *Mol. Cell. Endocrinol. **13**, 213.*

Simpson, E.R., Ackerman, G.E., Smith, M.E., Mendlson, C.R. (1981). *Proc. Natl Acad. Sci. USA **78**, 5690.*

Simpson, E.R., Graham-Lorence, S., Corbin, C.J., Merrill, J.C., Mahendroo, M.S. & Mendelson, C.R. (1989). *Horm. Cell Regul. **196**, 49.*

Simpson, E.R., Masson, J.I., Mendelson, C.R., Estabrook, R.W. & Waterman, M.R. (1988). *In: The molecular and cellular endocrinology of the testis Vol. **50**, pp 73-84, Eds B.A. Cooke and R.M. Sharpe. Raven press.*

Simpson, E.R., Merrill, J.C., Hollub, A.J., Graham-Lorence, S & Mendelson, C.R. (1989). *Endocr. Rev. **10**, 136.*

Simpson, E.R., Lund, J., Ahlgren, R. & Waterman, M.R. (1990). *Mol. Cell. Endocrinol. **70**, C25.*

Skakkeback, N.E., Berthelsen, J.G. & Müller, J. (1984). *Rec. Prog. Cell. Endocrinol. Testis INSERM **123**, 483.*

Skarin, G., Nillius, S.J., Wibell, L. & Wide, L. (1982). *J. Clin. Endocrinol. Metab. **55**, 723.*

Skinner, M.K. (1991). *Endocr. Rev. **12**, 45.*

Skinner, M.K., Fetterolf, P.M. & Anthony, C.T. (1988). *J. Biol. Chem. **263**, 2884.*

Skinner, M.K. & Fritz, I.B. (1985)a. *Proc. Natl. Acad. Sci. USA **82**, 114.*

Skinner, M.K. & Fritz I.B. (1985)b. *Mol. Cell. Endocrinol. **40**, 115.*

Skinner, M.K., McLachlan, R.I. & Bremner, W.J. (1989)b. *Mol. Cell. Endocrinol. **66**, 239.*

Skinner, M.K., Takacs, K. & Coffey, R.J. (1989)a. *Endocrinology **124**, 845.*

Slamon, D.J. & Cline, M.J. (1984). *Proc. Natl. Acad. Sci. USA **81**, 7141.*

Slamon, D.J., deKernion, J.B., Verma, I.M. & Cline, M.J. (1984). *Science* ***224,*** *256.*

Smith, E.P., Svoboda, M.E., van Wyk, J.J., Kierszenbaum, A.L., Tres, L.L. (1987). *Endocrinology* ***120,*** *186.*

Smith, E.R., Johnson, J., Weick, R.F., Levine, S. & Davidson, J.M. (1971). *Neuroendocrinology* ***8,*** *94.*

Smith, M.S. & Bartke, A.J. (1987). *Biol. Reprod.* ***36,*** *138.*

Smith, P.E. (1930). *Am. J. Anat.* ***45,*** *205.*

Smith, W.L. (1989). *Biochem. J.* ***259,*** *315.*

Smythies, J.R. (1988). *In Prostaglandins: biology and chemistry of prostaglandins and related eicosanoids, pp 631-638. Ed P.B. Curtis-Prior. Churchill Livingstone: Edinburgh-London-Melbourne-New York.*

Snell, K.C. (1965). *In: The pathology of laboratory animals, pp 241-300, W.E. Ribelin & J.R. McCoy (eds). Spring field: Thomas.*

Sokoloff, P., Giros, B., Martres, M.-P., Bouthenet, M.-L. & Schwartz, J.-C. (1990). *Nature* ***347,*** *146.*

Sporn, M.B. & Roberts, A.B. (1985). *Nature* ***313,*** *745.*

Sprengel, R., Braun, T., Nikolics, K., Segaloff, D.L., Seeburg, P.H. (1990). *Mol. Endocrinol.* ***4,*** *525.*

Spruance, S.L., Wilcox, B., Richards, O.C., Foster, D.N., Huseby, R.A. & Samuels, L.T. (1978). *Cancer Res.* ***38,*** *424.*

Ssewannyana, E. & Lincoln, G.A. (1990). *J. Endocrinol.* ***127,*** *461.*

Steel, C.M. (1989). *The Lancet* ***2,*** *30.*

Steinberger, A. (1973). *Biol. Reprod.* ***6,*** *61.*

Steiner, R.A., Bremner, W.J. & Clifton, D.K. (1982). *Endocrinology* ***111,*** *2055.*

Steiner, A.L., Parker, C.W. & Kipnis, D.M. (1972). *J. Biol. Chem.* ***247,*** *1106.*

Steinschneider, A., McLean, M.P., Billheimer, J.T., Azhar, S. & Gibori, G. (1989). *Endocrinology* ***125,*** *569.*

Stevens, V.L., Tribble, D.L., Lambeth, J.D. (1985). *Arch. Biochem. Biophys.* ***242, 324.***

Stewart, M. & Stewart, F. (1977). *J. Mol. Biol.* ***116, 175.***

Stocco, D.M. & Kilgore, M.W. (1988). *Biochem. J.* ***249, 95.***

Stone, N.N., Fair, W.R. & Fishman, J. (1986). *The Prostate* ***9, 311.***

Stoof, J.C. & Kebabian, J.W. (1984). *Life Science* ***35, 2281.***

Strickland, T.W. & Puett, D. (1981). *Endocrinology* ***109, 1933.***

Strobl, F.J., Gilmore, C.A. & Levine, J.E. (1989). *Endocrinology* ***124, 1140.***

Strott, C.A. (1990). *Trends Endocrinol. Metab.* ***1, 312.***

Strulovici, B. & Lefkowitz, R.J. (1984). *J. Biol. Chem.* ***259, 4389.***

Styles, J.A., Kelly, M., Pritchard, N.R. & Elcombe, C.R. (1988). *Carcinogenesis* ***9, 1647.***

Sullivan, M.H.F., Abayasekara, D.R.E. & Cooke, B.A. (1988). *In: The molecular and cellular endocrinology of the testis. B.A. Cooke & R.M. Sharpe (eds). Serono Symposia. Raven Press.*

Sullivan, M.H.F. & Cooke, B.A. (1983). *Biochem. J.* ***216, 747.***

Sullivan, M.H.F. & Cooke, B.A. (1985). *Biochem. J.* ***230, 821.***

Sullivan, M.H.F. & Cooke, B.A. (1986). *Biochem. J.* ***236, 45.***

Suter, D.E. & Schwarts, N.B. (1985). *Endocrinology* ***117, 849.***

Suzuki, K. & Kimura, T. (1965). *Biochim. Biophys. Res. Commun.* ***19, 340.***

Swerdloff, R.S., Walsh, P.C., Jacobs, H.S. & Odell, W.D. (1971). *Endocrinology* ***88, 120.***

Swerdlow, A.J., Huttly, S.R.A. & Smith, P.G. (1987). *Br. J. Cancer* ***55, 97.***

Swinnen, K., Cailleau, J., Heyns, W. & Verhoeven, G. (1990). *Endocrinology* ***126, 142.***

Syed, V., Khan, S.A. & Ritzen, E.M. (1985). *Mol. Cell. Endocrinol.* ***40, 257.***

Takai, Y., Kikkawa, U., Kaibuchi, K. & Nishizuka, Y. (1984). In: Adv. Cyvl. Nucl. Prot. Phosph. Res. Vol 18, pp 119-158. P. Greengard & G.A. Robison (eds). Raven Press: New York.

Tahri-Joutei, A. & Pointis, G. (1988). *J. Reprod. Fert.* ***82, 247.***

Talmadge, K., Vamvakopoulos, N.C. & Fiddes, J.C. (1984). *Nature* ***307, 37.***

Tapanainen, J., Kuopio, T., Pelliniemi, L.J. & Huhtaniemi, I. (1984). *Biol. Reprod.* ***31, 1027.***

Taylor, S.S., Saraswat, L.D., Toner, J.A. & Bubis, J. (1986). *Ann. N.Y. Acad. Sci.* ***478, 191.***

Teerds, K.J., Closset, J., Rommerts, F.F.G., de Rooij, D.G., Stocco, D.M., Colenbrander, B., Wensing, C.J.G. & Hennen, G. (1989)c. *J. Endocrinol.* ***120, 97.***

Teerds, K.J., de Rooij, D.C., Rommerts, F.F.G. & Wensing, C.J.G. (1988).*J. Androl.* ***9, 343.***

Teerds, K.J., de Rooij, D.G., Rommerts, F.F.G., van den Hurt, R. & Wensing, C.J.G. (1989)b. *J. Endocrinol.* ***122, 689.***

Teerds, K.J., de Rooij, D.G., de Jong, F.H. & Rommerts, F.G. (1991) *J. Androl.* ***12, 171.***

Teerds, K.J., de Rooij, D.G., Rommerts, F.F.G., van der Tweel, I. & Wensing, C.J.G. (1989)d. *Arch. Androl.* ***23, 105.***

Teerds, K.J., Rommerts, F.F.G., van de Kant, H.J.G. & de Rooij, D.G. (1989)a. *J. Reprod. Fert.* ***87, 141.***

Terrier, P., Sheng, Z.-M., Schlumberger, M., Tubiana, M., Caillou, B., Travagli, J.-P., Fragu, P., Parmentier, C. & Riou, G. (1988). *Br. J. Cancer* ***57, 43.***

Thanki, K.H. & Steinberger, A. (1976). Endocr. *Res. Commun.* ***3, 49.***

Themmen, A.P.N., Hoogerbrugge, J.W., Rommerts, F.F.G. & van der Molen, H.J. (1985). *Biochim. Biophys. Res. Commun.* ***128, 1164.***

Themmen, A.P.N., Hoogerbrugge, J.W., Rommerts F.FG. & van der Molen, H.J. (1986). *J. Endocrinol.* ***108, 431.***

Thompson, E.A. Jr & Siiteri, P.K. (1974)a. *J. Biol. Chem.* ***249, 5364.***

Thompson, E.A. Jr, Siiteri, P.K. (1974)b. *J. Biol. Chem.* ***249, 5373.***

Thompson, S.W., Huseby, R.A., Fox, M.A., Davis, C.L. & Hunt, R.D. (1961). *J. Natl Cancer Inst.* ***27, 1037.***

Thorell, J. I. & Johansson, B. G. (1971). *Biochim. Biophys. Acta* ***251, 363.***

Tilson-Mallett, N., Sautner, S.J., Feil, P.D. & Sauten, R.J. (1983). *J. Clin. Endocrinol. Metab.* ***57, 1125.***

Tindal, D.J., Miller, D.A. & Mean, A.R. (1977). *Endocrinology* ***101, 13.***
Toaff, M.E., Strauss, J.F. & Hamond, J.M. (1983). *Endocrinology* ***112, 1156.***

Tong, Y., Simard, J., Labrie, C., Zhao, H.F., Labrie, F. & Pelletier, G. (1989). *Endocrinology* ***125, 1821.***

Townsley, J. & Brodie, H.J. (1968). *Biochemistry* ***7, 33.***

Travali, S., Koniecki, J., Petralia, S. & Baserga, R. (1990). *FASEB J.* ***4, 3209.***

Tremblay, Y., Ringler, G.E., Morel, Y., Mohandas, T.K., Labrie, F., Strauss, J.F. & Millerm W.L. (1989). *J. Biol. Chem.* ***264, 20458.***

Tresguerres, J.A.F., Esquifino, A.I., Perez-Mendez, L.F. & Lopez-Calderon, A. (1981). *Endocrinology* ***108, 83.***

Tsai, S.C., Ying, B.P. & Gaylor, J. (1964). *Arch. Biochim. Biophys.* ***105, 329.***

Tsai-Morris, C.H., Aquilano, D.R. & Dufau, M.L. (1984). *Ann. N.Y. Acad. Sci.* ***438, 666.***

Tsai-Morris, C.-H., Aquilano, D.R. & Dufau, M.L. (1985). *Endocrinology* ***116, 38.***

Tsai-Morris, C.H., Beuzko, E., Wang, W. & Dufau, M.L. (1990). *J. Biol. Chem.* ***32, 19385.***

Tsai-Morris, C.-H., Knox, G., Luna, S. & Dufau, M.L. (1986). *J. Biol. Chem.* ***261, 3471.***

Tsai-Morris, C.-H., Knox, G.F. & Dufau, M.L. (1988). *J. steroid Biochem.* ***29, 285.***

Tsujimura, T. & Matsumoto, K. (1979). *J. Natl Cancer Inst.* ***63, 969.***

Ueno, H. & Mori, H. (1990). *Biol. Reprod.* ***43, 769.***

Ulisse, S., Fabbri, A. & Dufau, M.L. (19889). *J. Biol. Chem.* ***264, 2156.***

Ulisse, S., Fabbri, A., Tinajero, J.C. & Dufau, M.L. (1990). *J. Biol. Chem.* ***265, 1964.***

Urban, R.J., Garmey, G.C., Shupnik,, M.A. & Valdhuis, J.D. (1990). *Endocrinology* ***127, 2481.***

Urbanski, H.F., Pickle, R.L., Ramirez, V.D. (1988). *Endocrinology* ***123, 413.***

Vahouny, G.V., Chanderbahn, R., Noland, B.J., Irwin, D., Drnnis, P., Lambeth, J.D. & Scallen, T.J. (1983). *J. Biol. Chem.* ***258, 11731.***

Valensi, P., Coussieu, C., Pauwels, A., Atttali, J.R., Kemeny, J.L., Amouroux, J. & Sebaoun, J. (1987). *J. Endocrinol. Imvest.* ***10, 187.***

Valladares, L.E. & Payne, A.H. (1979)a. *Endocrinology* ***105, 431.***

Valladares, L.E. & Payne, A.H. (1979)b. *Proc. Natl. Acad. Sci.* ***76, 4460.***

Vallar, L., Vicentini, LML & Meldolesi, J. (1988). *J. Biol. Chem.* ***263, 10127.***

Vallar, L. & Meldolesi, J. (1989). *TIPS* ***10, 74.***

Vallar, L., Spada, A. & Ciannattasio, G. (1987). *Nature* ***330, 566.***

Valenca, M.M. & Negro-Vilar, A. (1986). *Endocrinology* ***118, 32.***

Vance, M.L., Cragun, J.R., Reimnitz, C., Chang, R.J., Rachef, E., Blackwell, R.E., Miller, M.M. & Molitch, M.E. (1989).. *J. Clin. Endocrinol. Metab.* ***68, 336.***

van der Burg, B. van Selm-miltenburg, A.J.P., de Laat, S.W. & van Zoelen, E.J.J. (1989). *Mol. Cell. Endocrinol.* ***64, 223.***

van der Molen, H.J., Bijleveld, M.J., van dier Vusse, G.J. & Cooke, B.A. (1972). *J. Endocrinol.* ***57, 6.***

van der Molen, H.J. & Rommerts, F.F.G. (1981). *In: The testis, pp 213-237, Eds H. Burger and D. de Kretser. Raven Press: New York.*

van der Vusse, G.J., Kalkman, M.L. & van der Molen, H.J. (1974). *Biochim. Biophys. Acta* ***348, 404.***

van der Vusse, G.J., Kalkman, M.L. & van der Molen, H.J. (1975). *Biochim. Biophys. Acta* ***380, 473.***

vanDongen, AMJ, Codina, J., Olate, J., Mattera, R., Joho, R., Birnbaumer, L. & Brown, A.M. (1988). *Science **242, 1433-1437.***

van Noort, M., Rommerts, F.F.G., van Amerongen, A. & Wirtz, K.W.A. (1986). *J. Endocrinol. **109, R13.***

van Noort, M., Rommerts, F.F.G., van Amerogen, A. & Wirtz, K.W.A. (1988). *Biochim. Biophys. Res. Commun. **154, 60.***

van Thiel, D.H., Gavaler, J.S., Smith, W.I. & Paul, G. (1979). *N. Eng. J. Med. **300, 112.***

Velu, T.J., Martin, P., Vass, W.C., Helin, K., Ritzhaupt, A., Beguinot, L., Schiller, J.T. & Lowry, D.R. (1989). *Horm. Cell Regul. **198, 65.***

Velu, T.J. (1990). *Mol. Cell. Endocrinol. **70, 205.***

Verhoeven, G. & Cailleau, J. (1986)a. *J. Steroid Biochem. **25, 393.***

Verhoeven, G. & Cailleau, J. (1986)b. *Mol. Cell. Endocrinol. **47, 99.***

Verhoeven, G. & Cailleau, J. (1987). *Mol. Cell. Endocrinol. **49, 137.***

Verhoevan, G. & Cailleau, J. (1988). *Endocrinology **123, 2100-2110.***

Verjans, H. L., Cooke, B. A., de Jong, F. H., de Jons, C. M. M. & van der Molen, H. J. (1973). *J. Steroid Biochem. **4, 665.***

Vidal, M.E., Davis, J.R.E., Wilson, E.M. & Sheppard, M.C. (1988). *J. Endocrinol. **117 (Suppl.), Abstract 251.***

Vihko, K.K. & Huhtaniemi, L. (1989). *Mol. Cell. Endocrinol. **65, 119.***

Vijayan, E. & Mc Cann, S.M. (1978). *Neuroendocrinology **25, 150-165.***

Vinggaard, A.M. & Hansen, H.S. (1991). *Mol. Cell. Endocrinol. **79, 157.***

Vircburger, M.I., Prelevic, G.M., Brkic, S., Andrejevic, M.M. & Peric, G.A. (1981). *Lancet **1, 1160.***

Virmani, M.A., Stojilkovic, S.S. & Catt, K.J. (1990). *Endocrinology **126, 2499.***

Voutilainen, R. & Miller, W.L. (1986). *J. Clin. Endocrinol. Metab. **63, 1145.***

Wallis, M., Howell, S.L. & Taylor, K.W. (1985). *In: The biochemistry of the polypeptide hormones, pp 147-183. John Wiley and Sons: New York.*

Walters, J.R., Bergstrom, D.A., Carlson, J.H., Chase, T.N. & Braun, A.R. (1987). *Science* ***236, 719.***

Walters, J.R., Juniewicz, P.E., Oesterling, J.E., Chamindrani Mendis-Handagama, S.M.L., Zirkin, B.R. & Ewing, L.L. (1988). *Endocrinology* ***123, 2223.***

Wang, H., Ascoli, M. & Segaloff, D.L. (1991)a. *Endocrinology* ***129, 133.***

Wang, H., Segaloff, D.L. & Ascoli, M. (1991)b. *Endocrinology* ***128, 2651.***

Wang, N.-G., Sundaram, K., Pavlou, S., Rivier, J., Vale, W. & Bardin, C.W. (1983). *Endocrinology* ***112, 331.***

Waterman, M.R. & Simpson, E.R. (1985). *Mol. Cell. Endocrinol.* ***39, 81.***

Watson, J.V., Stewart, J., Evan, G.I., Ritson, A. & Sikora, K. (1986). *Br J. Cancer* ***53, 331.***

Waxman, J., Lowe, D., Whitfield, H.N., Hendry, B.F. & Besser, G.M. (1987). *Fert. Steril.* ***48, 1067.***

Wee, S. & Grogan, W.M. (1989). *Lipids* ***24, 824.***

Weeler, M.B. & Veldhuis, T.D. (1989). *Endocrinology* ***125, 2414.***

Weinberg, R.A. (1985). *Science* ***230, 770.***

Weinstein, R.L., Kelch, R.P., Jenner, M.R., Kaplan, S.L. & Grumbach, M.M. (1974). *J. Clin. Invest.* ***53, 1.***

Welsh, T.H., Bambino, T.H. & Hsueh, A.J.W. (1982). *Biol. Reprod.* ***27, 1138.***

Welsh, T.H. & Hsueh, A.J.W. (1982). *Endocrinology* ***110, 1498.***

Weniger, J.-P. & Zeis, A. (1988). *J. Endocrinol.* ***118, 485.***

West, A.P. & Cooke, B.A. (1991) *Endocrinology* ***128, 363.***

West, R. & Dannies, P.S. (1980). *Endocrinology* ***106, 1108.***

Whitehouse, B.J. (1989). *J. Endocrinol.* ***123, 363.***

Widmark, A., Damber, J.E. & Bergh, A. (1986). *J. Endocrinol.* ***109, 419.***

Widmark, A., Damber, J.E. & Bergh, A. (1987). *J. Endocrinology* ***115, 489.***

Wiemann, J.N., Clifton, D.K. & Steiner, R.A. (1990). *Endocrinology **127, 523.***

Wierman, M.E., Rivier, J. & Wang, C. (1989). *Endocrinology **124, 272.***

Wilding, G., Zugmeier, G., Knabbe, C., Flanders, K. & Gelmann, E. (1989). *Mol. Cell. Endocrinol. **62, 79.***

Wilkinson, M., Moger, W. & Grovestine, D. (1980). Life Science **27**, 2285.

Wilson, S. P. (1988). *Second Messengers and Phosphoproteins **12, 1.***
Xie, Y.B., Wang, H., Segaloff, D.L. (1990). *J. Biol. Chem. **265, 21411.***

Yanagibashi, K., Ohno, Y., Kawamura, M. & Hall, P.F. (1988). *Endocrinology **123, 2075.***

Yatani, A., Codina, J., Imoto, Y., Reeves, J.P., Birnbaumer, L. & Brown, A.M. (1987). *Science **238, 1288.***

Yatani, A., Imoto, Y., Codina, J., Hamilton, S.L., Brown, A.M. & Birnbaumer, L. (1988). *J. Biol. Chem. **263, 9887.***

Yee, J. B. & Hutson, J. C. (1983). *Biol. Reprod. 29, 1319.*

Yee, J. B. & Hutson, J. C. (1985)a. *Biol. Reprod. **32, 872.***

Yee, J. B. & Hutson, J. C. (1985)b. *Endocrinology **116, 2682.***

Yoon, D.J., Skiar, C. & David, R. (1988). *Endocrinology **122, 759.***

Yoshimasa, T., Sibley, D.R., Bouvier, M., Lefkowitz, R.J. & Caron, M.G. (1987). *Nature **327, 67.***

Yoshizaki, K., Matsumoto, K. & Samuels, L.T. (1978). *Endocrinology **102, 918.***

Zimniski, S.J., Melner, M.H. & Puett, D. (1985). *Endocrinology **116, 2583.***

Zipf, W.B., Payne, A.H. & Kelch, R.P. (1978). *Biochim. Biophys. Acta **540, 330.***

Zirkin, B.R. & Ewing, L.L. (1987). *Anat. Rec. 219, 157.*

Zuber, M.X., John, M.E., Okamura, T., Simpson, E.R. 7 Waterman, M.R. (1986). *Science **234, 1258.***

PUBLICATIONS AND COMMUNICATIONS

Dirami, G., Poulter, L.W. & Cooke, B.A (1988). Separation and characterization of macrophages and Leydig cells from the rat testes. *J. Endocrinol.* ***119*** *(Suppl) Abstract 132.*

Dirami, G., Poulter, L.W. & Cooke, B.A. (1990). Removal of macrophages from Leydig cell preparations using centrifugal elutriation, density gradient centrifugation and Dynabeads coated with a macrophage monoclonal antibody. Sixth Europeen Worshop on Molecular and Cellular Endocrinology of the Testis, Mariehamn, Finland, 23-26 May. Abstract F10.

Dirami, G., Teerds, K.A & Cooke, B.A. (1991). Effects of a dopamine agonist on the function and growth of Leydig cells in rat testes. J. Reproduction and Fertility, SSF Annual Conference, Oxford, July 1991, Abstract 7.

Dirami, G., Poulter, L.W. & Cooke, B.A. (1991). Separation and characterization of Leydig cells and macrophages from rat testes. *J. Endocrinology* ***130,*** *357-365.*

Cooke, B.A., Dirami, G., Chaudry, L., Choi, M.S.K., Abayasekara, D.R.E. & Phipp, L. (1991). Release of arachidonic acid and the effects of corticosteroids on steroidogenesis in rat testis Leydig cells. *J. Steroid Biochem. Molec. Biol.* ***40,*** *465-471.*