Hormones and the control of biochemical processes in spermatogenesis

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door
Johan Anton Grootegoed
geboren te Amsterdam

Promotor : Prof. Dr. H.J. van der Molen

Coreferenten : Prof. Dr. H. Galjaard
Dr. M.F. Kramer

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APPENDIX PAPERS

Paper 1

J.A. Grootegoed, M.J. Peters, E. Mulder, F.F.G. Rommerts and H.J. van der Molen. Absence of a nuclear androgen receptor in isolated germinal cells of rat testis. Molecular and Cellular Endocrinology 9 (1977) 159-167.

Paper 2

J.A. Grootegoed, A.H. Grollé-Hey, F.F.G. Rommerts and H.J. van der Molen. Ribonucleic acid synthesis in vitro in primary spermatocytes isolated from rat testis. Biochemical Journal 168 (1977) 23-31.

Paper 3

J.A. Grootegoed, L.M. van Meerkerk, F.F.G. Rommerts and H.J. van der Molen. Ribonucleic acid synthesis in vitro in primary spermatocytes isolated from testes of intact and hypophysectomized rats. Submitted for publication.

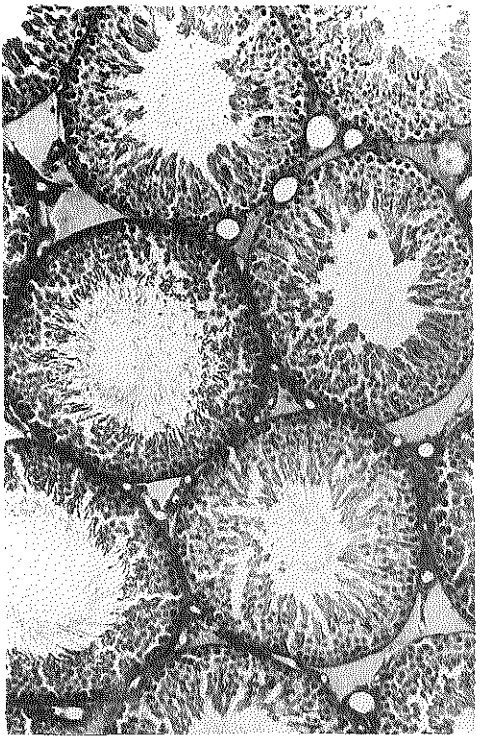
Chapter 1

INTRODUCTION

The aim of the experiments described in this thesis was to contribute to a better understanding of the relationship, in molecular terms, between hormones and the development of male germinal cells in rats. In this introduction we will discuss some important aspects of spermatogenesis, the hormonal control of spermatogenesis, and the aim and scope of the present thesis.

1.1 Spermatogenesis

Spermatogenesis is the formation of spermatozoa which, in mammals, occurs in the seminiferous tubules of the testis (Fig. 1). The spermatozoa are the final product of mitotic and meiotic divisions as well as the expression of cellular differentiation (Fig. 2). Spermatogenesis in the rat starts with the appearance of type A spermatogonia from precursor cells, at the fourth postnatal day (20). The type A spermatogonia undergo a complex series of mitotic divisions and give rise to type B spermatogonia and a new generation of type A spermatogonia (16,152). Primary spermatocytes are formed after mitotic divisions of type B spermatogonia. In the primary spermatocytes the DNA is doubled during the so-called preleptotene stage. Subsequently the spermatocytes enter a long meiotic prophase which, based on the morphology of the condensed chromosomes, is subdivided in a leptotene, zygotene, pachytene and diplotene stage (21). During this meiotic prophase the homologous chromosomes are paired and exchange genetic information. The first meiotic division, which takes place after completion of the prophase, gives rise to secondary spermatocytes which rapidly undergo the second meiotic division and haploid spermatids are formed. The spermatids do not divide but are transformed further via several interme-



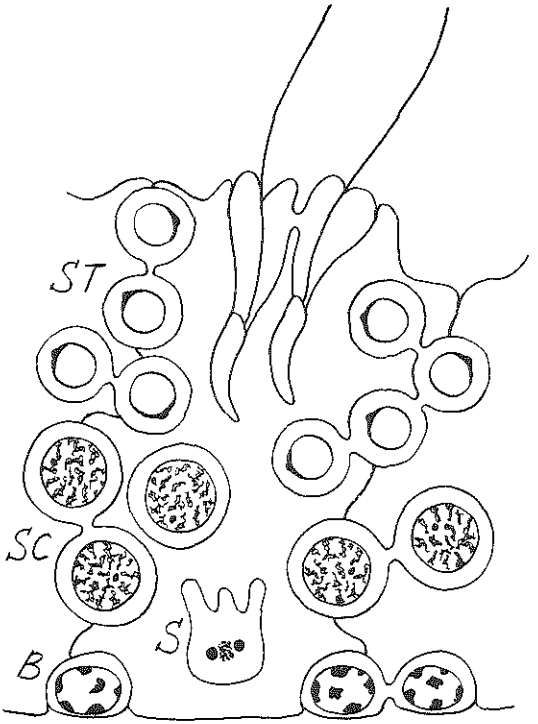


Figure 1a Histology of testicular tissue of an adult rat (fixed with glutaraldehyde and stained with periodic acid Schiff and hematoxylin, X 100). The cylindrical seminiferous epithelium, with the germinal cells at different stages of development and the Sertoli cells, is surrounded by the tubular wall with myoid cells and endothelial cells (34). The interstitial space between the tubules is occupied by the hormone producing Leydig cells, blood and lymphatic vessels (49).

Figure 1b Schematic drawing of a part of the seminiferous epithelium. The cytoplasm of the Sertoli cells surrounds the germinal cells at different steps of development.

S - Sertoli cell nucleus
B - type B spermatogonia
SC - pachytene spermatocytes
ST - round and elongating spermatids

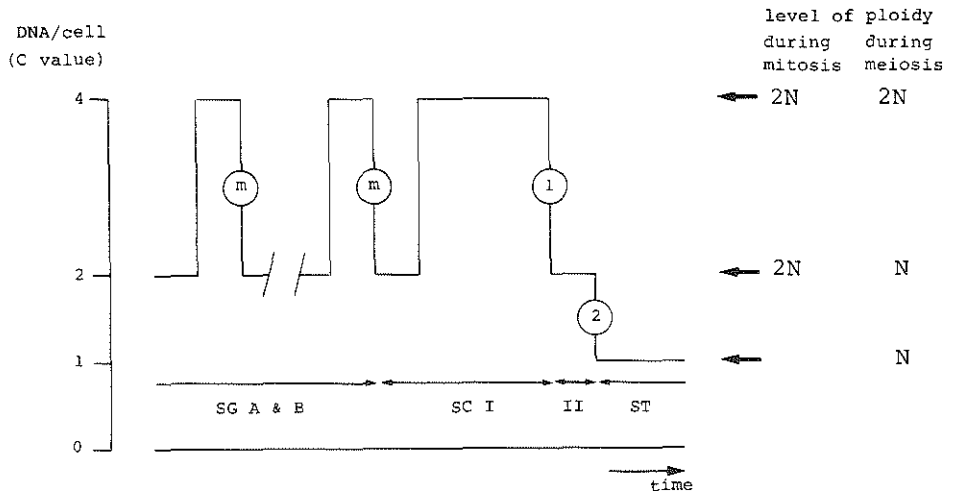


Figure 2 Scheme of mitosis and meiosis during spermatogenesis in the rat. The amount of DNA per cell (C represents the amount of DNA in a gamete) and the level of ploidy (single set of chromosomes or haploid = N, double set of chromosomes or diploid = 2N) during spermatogenesis are schematized in this figure. The amount of DNA in the diploid spermatogonia and preleptotene spermatocytes is doubled before the start of the mitotic or meiotic prophase respectively. During the mitotic divisions of spermatogonia the two chromatids of each chromosome move to opposite poles, and the level of ploidy is not changed whereas the amount of DNA is equally distributed over two cells. In contrast, the first meiotic division involves the separation of the homologous chromosomes. As a result the level of ploidy as well as the amount of DNA per cell is halved. The two chromatids of each chromosome are separated during the subsequent second meiotic division, which is not preceded by a period of DNA synthesis, and spermatids are formed which contain a single set of chromosomes and an amount of DNA which is equal to C.

m - mitotic divisions
1 and 2 - first and second meiotic division
SG A&B - A and B spermatogonia
SC I-II - primary and secondary spermatocytes
ST - spermatids

diate steps into spermatozoa. Type A spermatogonia will continuously produce new generations of type B spermatogonia so that a constant production of spermatozoa is achieved (16). The duration of spermatogenesis (from the divisions of type A_1 spermatogonia up to the release of spermatozoa from the Sertoli cells; 17) is approximately 50 days in the rat and 64 days in man (17,21).

During their development the germinal cells are connected to each other by cytoplasmic bridges (35) and they are closely associated with the somatic Sertoli cells in the seminiferous epithelium (Fig. 1). Adjacent Sertoli cells are interconnected via tight junctions, forming a blood-testis barrier for the penetration of macromolecules through the seminiferous epithelium (34). This barrier divides the tubules in a basal compartment, containing the spermatogonial population and early spermatocytes, and an adluminal compartment, which contains the more mature germinal cells (34). It has been shown that the composition of the fluid in the tubules at the adluminal side of the barrier is different from the composition of blood plasma with respect to ions and proteins (88,93,185).

The spermatogonia, spermatocytes and spermatids at various steps of development do not occur in random combinations in the seminiferous epithelium but appear to be present in specific associations. In the rat, 14 of such associations, called stages of the cycle of the seminiferous epithelium, have been described (15,91). These stages are found at successive well defined time intervals, in a given area of the tubules.

1.2 Effects of hormones on spermatogenesis

Spermatogenesis in the rat is dependent on the presence of peptide hormones from the pituitary. This has been shown in a quantitative morphological study by Clermont & Morgentaler (19), who observed that after surgical removal of the pituitary (hypophysectomy) extensive degeneration of germinal cells resulted in a decreased number of type A and B spermato-

gonia and early spermatocytes and a drastically decreased number of pachytene spermatocytes and spermatids. The rate of germ cell development, however, appears not to be influenced by hormones (17).

The pituitary hormone lutropin (LH) regulates the production of testosterone in the testicular Leydig cells (39). Spermatogenesis in hypophysectomized adult rats can be maintained by administration of testosterone or its biologically active metabolite dihydrotestosterone (110) when administration of these hormones is started immediately after hypophysectomy (1,18). From such observations it has been concluded that testosterone is the main hormone which is needed for maintenance of germinal cell development. The pituitary hormone follitropin (FSH) seems to be particularly important for spermatogenesis during the development of the testis in immature rats (109) as well as for restoration of spermatogenesis after regression due to the prolonged absence of hormones (101,102).

Steinberger (174) has proposed a model for the qualitative effects of hormones during spermatogenesis. In that model testosterone and follitropin influence spermatogenesis at several specific points. Relevant to this, Russel & Clermont (155) have suggested that, shortly after hypophysectomy of adult rats, the degeneration of pachytene spermatocytes and spermatids is enhanced specifically at stage VII of the cycle of the seminiferous epithelium.

Recent information strongly suggests that the Sertoli cells are target cells for follitropin and testosterone and that these hormones cannot interact directly with germinal cells (chapter 3). The development of the germinal cells is therefore possibly dependent on the action of hormones on the Sertoli cells.

1.3 Aim and scope of the present thesis

The aim of the present study was to gain insight in the biochemical processes in germinal cells of the male rat which might be involved in the action of hormones on the seminiferous epithelium.

A biochemical description of processes which may regulate the development of male germinal cells of mammals was hardly available. Steinberger commented in 1971 that "Knowledge of the biochemical parameters of the hormonal action on spermatogenesis is too meagre even for a limited attempt to formulate a working hypothesis" (174). Therefore we decided to study biochemical properties of germinal cells preliminary to the possible hormonal regulation of these properties.

Pachytene spermatocytes are markedly sensitive to the absence of hormones in vivo (19,155) and it has been observed that spermatogenesis in testicular tissue in vitro is nearly always blocked at the level of pachytene spermatocytes (172, 173). Because of these observations we concentrated our experiments mainly on this germinal cell type.

In initial experiments we have tried to correlate changes in enzyme activities and protein synthesis in total testicular tissue with the presence or activity of pachytene spermatocytes (chapter 3). Biochemical properties of total testicular tissue, however, cannot easily be interpreted in terms of specific cell types. Therefore we decided to work with isolated germinal cells.

A method for separation of cell types of different sizes by velocity sedimentation at unit gravity (125) has been widely employed to obtain cell preparations enriched in different types of germinal cells from mammalian testes (chapter 2). This method has recently been used to study enzymes (58, 124), nuclear proteins (57,60,62,100,114,145) and the synthesis of RNA and DNA (55,56,75,123) in spermatocytes or spermatids.

For our work we have used cell preparations enriched in pachytene spermatocytes or round spermatids (chapter 2). With such preparations we have tried to detect receptor proteins for androgens in these germinal cells because the target cells for testosterone in the testis were still not established.

The main part of the present thesis concerns experiments on the incorporation of radioactively labelled uridine into RNA of pachytene spermatocytes (chapter 4). This incorporation was studied because it has been shown that, in different hormone-dependent systems, steroid or peptide hormones act via one or more of the processes involved in RNA synthesis (chapter 5). It might be possible that testosterone and follitropin act on the germinal cells, via the Sertoli cells, in an analogous manner by influencing RNA synthesis. In the last part of the work presented in this thesis we have therefore tried to study whether hormones or factors from the Sertoli cell influence the incorporation of labelled uridine into RNA in pachytene spermatocytes (chapter 5).

Chapter 2

MATERIALS AND METHODS

Most of the materials and methods used in the present experiments have been described in the appendix papers. Some general aspects of these methods are presented in this chapter. Specific details of methods used in some of the experiments are described for the individual experiments.

2.1 Animals

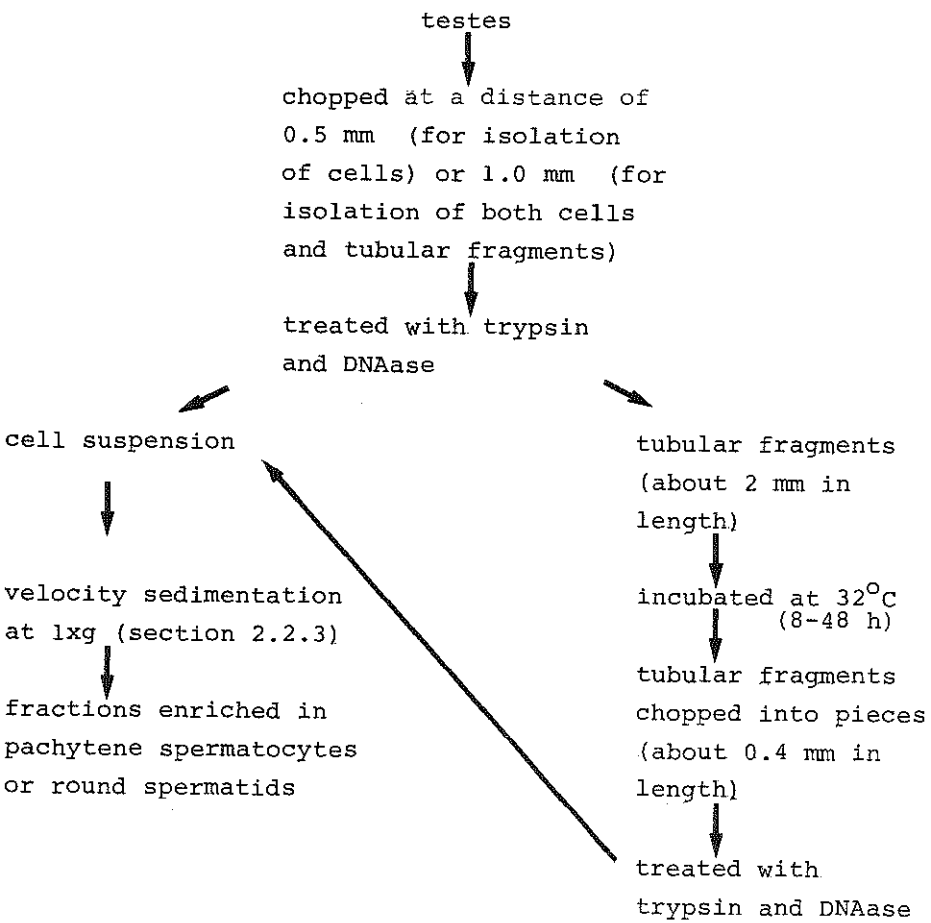
Rats were used for all experiments because morphological aspects of spermatogenesis have been well characterized in rats (21) and also much is known about the endocrine regulation of spermatogenesis in rats (174). The development of the testis and the first appearance of germinal cell types is related to the age and the body weight (128) of immature rats. In 26-28 day-old rats, spermatogenesis has reached the meiotic divisions and the first spermatids have been formed. In testes from 32-35 day-old rats a large number of spermatids (steps 1-8) are present and the first elongating spermatids have been formed. Immature rats (26-32 day-old) were used for most experiments because the development of spermatids is yet incomplete in these rats and therefore the number of round and elongating spermatids in isolated cell preparations of pachytene spermatocytes can be reduced (3,27, section 2.2.3). For several experiments, the pituitary gland of ether-anaesthetized rats was removed (hypophysectomy) with a Hoffman-Reiter hypophysectomy instrument. Injections of hormones (follitropin, testosterone) were given either subcutaneously or intraperitoneally and injection of radioactively labelled substances (^{3}H-leucine, ^{3}H-uridine, ^{3}H-testosterone) was performed either intratesticularly or intraveneously, as described for the individual experiments. Rats were killed by cervical dis-

location and testes were always removed immediately and decapsulated.

2.2 Isolation and incubation of testicular cells and tubular fragments

2.2.1 Scheme for isolation of germinal cells and tubular fragments

A schematic outline of the procedures used for the isolation of germinal cells and tubular fragments is given in the following scheme.



The details of the enzyme treatment and the isolation procedure have been presented in appendix paper 2.

Cell preparations which contain mainly Sertoli cells or peritubular cells have been obtained as described by Rommerts et al. (149).

2.2.2 Media used for isolation and incubation

Cells and tubular fragments were isolated in salt solutions, whereas the media used for incubation contained in addition amino acids and vitamins. A high K^+ concentration was used in media for the isolation and incubation of germinal cells and tubular fractions (Table I) because it has been shown that the concentration of K^+ is high in the tubular fluid in vivo (185). Sertoli cells and peritubular cells were isolated in Hank's Balanced Salt Solution (68) and incubated in Eagle's Minimum Essential Medium (37) as described by Rommerts et al. (149).

Incubations were performed at 32°C (the scrotal temperature of the rat testis) in an atmosphere of air/CO_2 (19:1) unless described otherwise.

2.2.3 Separation of germinal cell types by velocity sedimentation at unit gravity

The technique to isolate cells by velocity sedimentation at unit gravity (Staput method, 125,144) has been widely used to separate germinal cells from mammalian testes (13,27,56, 58,89,99,122,145,150). With this method it is possible to separate cells with approximately equal density on basis of their different sizes. Pachytene spermatocytes are larger than round spermatids and therefore sediment faster. The gradient of albumin or Ficoll which is formed underneath the cell suspension (Fig. 1) merely functions to prevent turbulent mixing. For the present experiments the separation was performed either at room temperature for 80 min or at 5°C for 100 min. After these time periods pachytene spermatocytes and

Table I Composition of the media used for isolation and incubation of germinal cells and tubular fragments.

	isolation medium	incubation medium
NaCl (mM)	69.8	69.8
KCl (mM)	85.8	56.9
$NaHCO_3$ (mM)	3.6	23.8
other salts	as present in Hank's Balanced Salt Solution (68)	as present in Earle's Balanced Salt Solution (38)
glucose (mM)	5.6	3.3
sodium-pyruvate (mM)	-	1
sodium-DL-lactate (mM)	-	6
essential amino acids and vitamins	-	60% of the amount as present in Eagle's Minimum Essential Medium (37)
non-essential amino acids (Gibco, 100 X) (ml/l)	-	10
L-glutamine (mM)	-	2
BSA (fraction V, B grade, Calbiochem) (g/l)	0-40	4
penicillin (i.u.)	10^5	10^5
streptomycin (mg/l)	100	100
fungizone (mg/l)	1	1
phenol red (mg/l)	10	6
pH	6.9-7.1 (in air)	7.0-7.2 (in 5% CO_2, 95% air)

round spermatids are separated (Fig. 1) so that both cell types can be collected in different fractions (Fig. 2 and appendix paper 2). However, a pure fraction of pachytene spermatocytes is not obtained because somatic cell types as well as multinucleated spermatogonia, early primary spermatocytes and spermatids cosediment with the pachytene spermatocytes (Table 2). The significance of these other cell types for the experiments on RNA synthesis in pachytene spermatocytes will

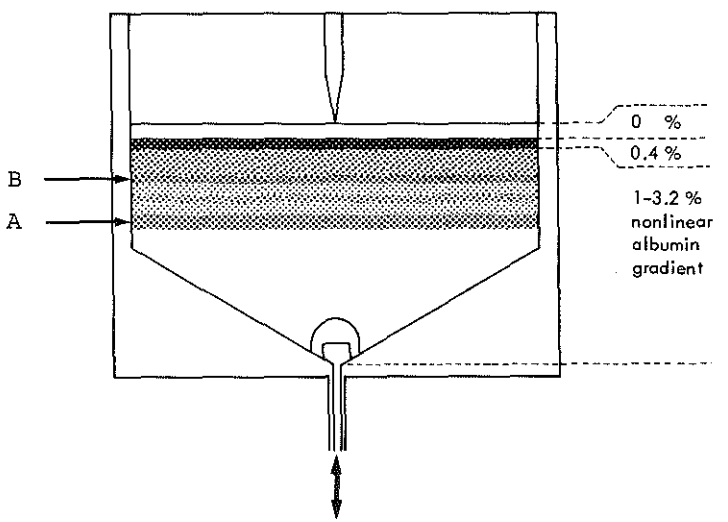


Figure 1 Cross section of the cylindrical sedimentation column used for separation of germinal cells. The sedimentation column is filled from the bottom successively with 60 ml of isolation medium and 24 ml of the cell suspension. Then the cells are lifted by a gradient of albumin (1-3.2%) in isolation medium. In the figure the column is shown after the cells have sedimented. Bands of cells can be seen which contain mainly pachytene spermatocytes (A) and, when rats of 32-35 days of age are used, round spermatids (B).

Table II Cellular composition of preparations of pachytene spermatocytes. Cell suspensions were prepared from total testicular tissue of 26-35 day-old rats (left column) and from tubular fragments isolated from testes of 26-32 day-old rats (right column). The tubular fragments were used for isolation of pachytene spermatocytes after 1 day of incubation. After cell separation by velocity sedimentation at unit gravity fractions enriched in pachytene spermatocytes were collected (fraction A in Figs. 1 and 2). The isolated cells were fixed in Bouin's fixative on microscope slides, stained with hematoxylin and eosin and identified as described by Platz et al. (145).

Cell type	Percentage (± S.D.) of testicular cell types in enriched preparations of pachytene spermatocytes isolated from:	
	total testicular tissue (n = 6)	tubular fragments (n = 3)
spermatogonia and preleptotene spermatocytes	0.1 ± 0.2	0.2 ± 0.3
idem, multinucleate	0.5 ± 0.4	0.5 ± 0.4
leptotene, zygotene and early pachytene spermatocytes	0.7 ± 0.7	4.0 ± 1.2
idem, multinucleate	1.6 ± 0.9	2.5 ± 1.0
middle- and late pachytene spermatocytes	68.9 ± 6.7	66.5 ± 9.5
idem, multinucleate	10.2 ± 5.1	9.5 ± 1.6
dividing cells and secondary spermatocytes	2.1 ± 1.1	1.6 ± 0.2
round spermatids (steps 1-7)	1.5 ± 1.6	0.4 ± 0.4
idem, multinucleate	9.5 ± 5.0	4.2 ± 3.1
Sertoli cells	0.9 ± 0.9	4.8 ± 1.9
other somatic cell types	1.4 ± 2.3	0.7 ± 0.5
cytoplasmic fragments	0.7 ± 0.4	0.2 ± 0.2
unknown and broken cells	1.9 ± 1.0	4.9 ± 1.3

be discussed in section 4.3.

The viability of the isolated cells has been estimated using the incorporation of ^{3}H-uridine into RNA (chapter 4). The ultrastructure of isolated spermatocytes and spermatids for most of the cells was found to be similar to that of comparable cells in testicular tissue.

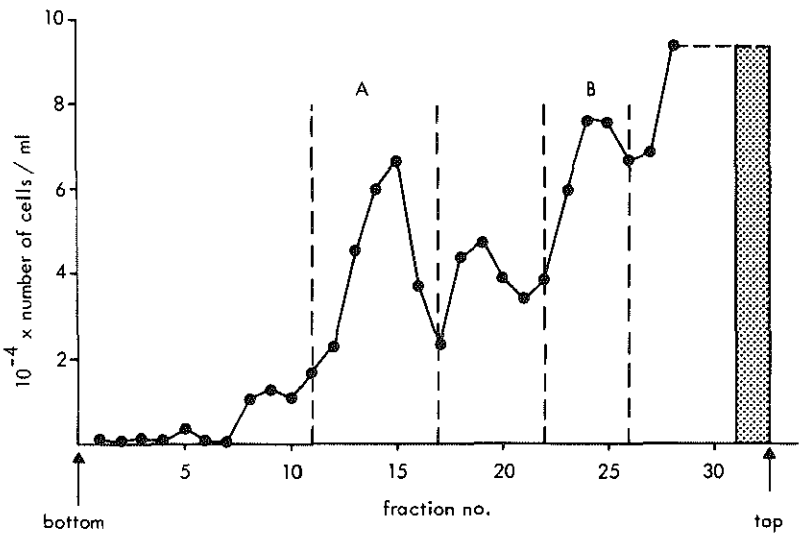


Figure 2 Distribution of cells in fractions collected from the sedimentation column. Fractions of 15 ml were collected from the bottom of the sedimentation column. The number of cells/ml in each fraction is shown after separation of cells obtained from isolated tubular fragments (26 day-old rats) which were incubated for 1 day. The top fractions contained damaged cells and nuclei and the cells in these fractions were therefore not counted. For routine fractionation of cells, region A (pachytene spermatocytes) and, when rats of 32-35 days of age were used, region B (round spermatids) were collected as single fractions.

2.3 Analysis of RNA synthesis

RNA synthesis was studied by analysis of the incorporation of radioactivity from (5-^{3}H)uridine, (2-^{14}C)uridine or L-(methyl-^{14}C)methionine into RNA. To obtain qualitative information on this incorporation, nucleic acids were extracted from cells or tissues after incubation with radioactive precursors. This extraction was performed with a two-phase mixture of phenol and aqueous salt solution. The phenol denatures the proteins and subsequently the nucleic acids are precipitated from the aqueous phase with ethanol (details of the method are described in appendix paper 2). Both cytoplas-

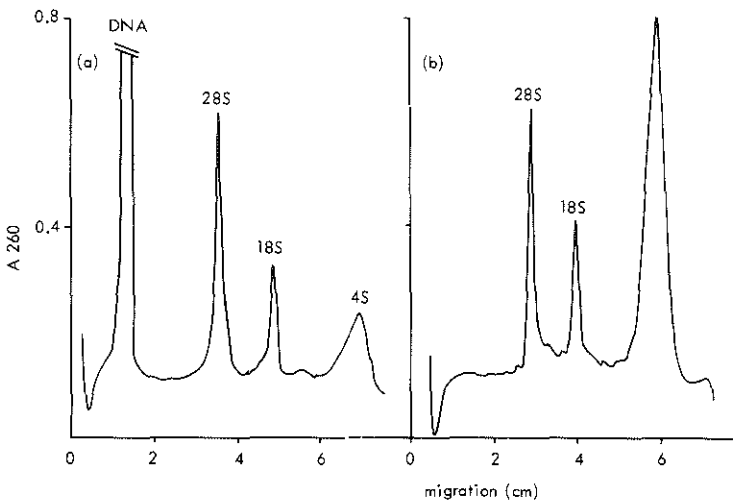


Figure 3 Profiles of the absorbance at 260 nm of nucleic acids after separation by electrophoresis in polyacrylamide gels (A_{260} profiles). Nucleic acids were extracted from tubular fragments and separated by electrophoresis in 2.4% polyacrylamide gels.

3a) Without DNAase treatment.

3b) DNA removed with DNAase (the absorption in the 4S region of the gel is increased due to the presence of the DNAase).

mic and nuclear nucleic acids are extracted, but the DNA is removed after treatment with DNAase (Fig. 3). The RNA is separated into different fractions by electrophoresis in polyacrylamide gels (appendix paper 2). The mass of the extracted RNA (28S and 18S rRNA and, when added as a carrier, 23S and 16S E.coli rRNA) is shown in the profiles of the absorption at 260 nm (Fig. 4). The profiles of the distribution

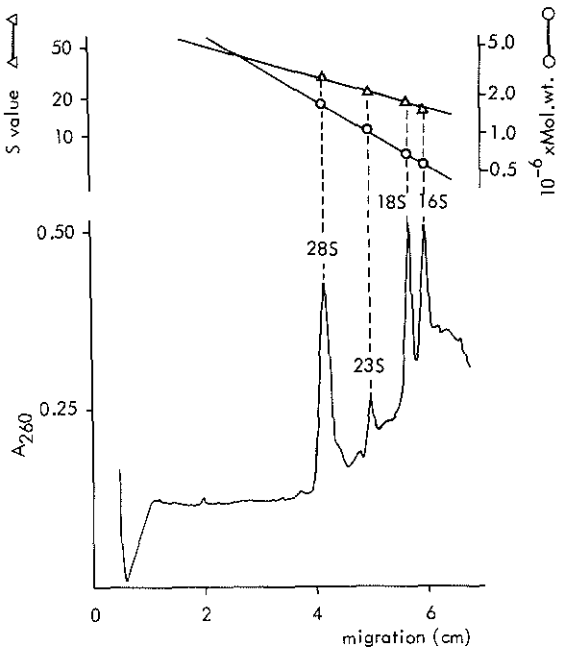


Figure 4 Migration of different RNA species during electrophoresis in polyacrylamide gels. RNA was extracted from isolated pachytene spermatocytes with E.coli 23S and 16S rRNA added as a carrier. The S value and molecular weight of the RNA components is plotted semi-logarithmically as a function of the migration during electrophoresis in the gel. A linear relation is found which makes it possible to calculate the S value and mol. wt. of unknown RNA components (95,97). The mol. wt. of rat liver 28S and 18S rRNA is 1.75- and 0.70×10^6 respectively (23), the mol.wt. of E.coli 23S and 16S rRNA is 1.08- and 0.56×10^6 respectively (170).

of radioactivity in the gels show which RNA components are labelled after incubation of the cells or tissues with radioactive precursors (Fig. 5). The molecular weight and S value of the labelled RNA components can be calculated using 28S, 18S, 23S and 16S RNA as markers (Figs. 4 and 5). In general, radioactivity from uridine is found in undefined peaks and a

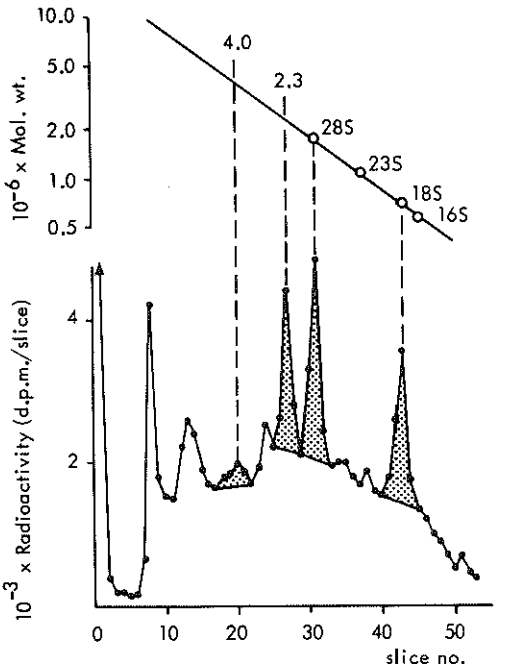


Figure 5 Profile of radioactively labelled RNA after electrophoresis in polyacrylamide gels. Isolated tubular fragments from 26 day-old rats were incubated for 8 h with ^{3}H-uridine (5 µCi/ml, specific radioactivity 29 Ci/mmol). RNA was extracted and was separated by electrophoresis in a polyacrylamide gel. The radioactivity in the non-shaded area of the profile is called "heterogeneous" RNA. The shaded areas represent RNA which was identified as presumptive rRNA. RNA peaks with a molecular weight of 2.3 and 4.0×10^{6} were present which may represent 32S and 45S rRNA respectively (the presence of the 32S rRNA peak is explained in chapter 4). The mol. wt. of rat liver 32S and 45S rRNA is approx. 2.2- and 4.6×10^{6} respectively (23).

heterogeneous background ("heterogeneous RNA") as well as in peaks which are identified as rRNA components (Fig. 5).

The cellular uptake as well as the incorporation of ^{3}H-uridine into RNA has also been analyzed in a quantitative manner. The uptake is defined as the radioactivity present in acid-soluble plus acid-precipitable material whereas the incorporation is equivalent to the radioactivity in acid-precipitable material (appendix paper 2).

Radioautography of the isolated cells after labelling with ^{3}H-uridine was used in order to evaluate the homogeneity of isolated cell preparations with respect to RNA synthetic capacity of individual cells (Fig. 6 and appendix paper 3).

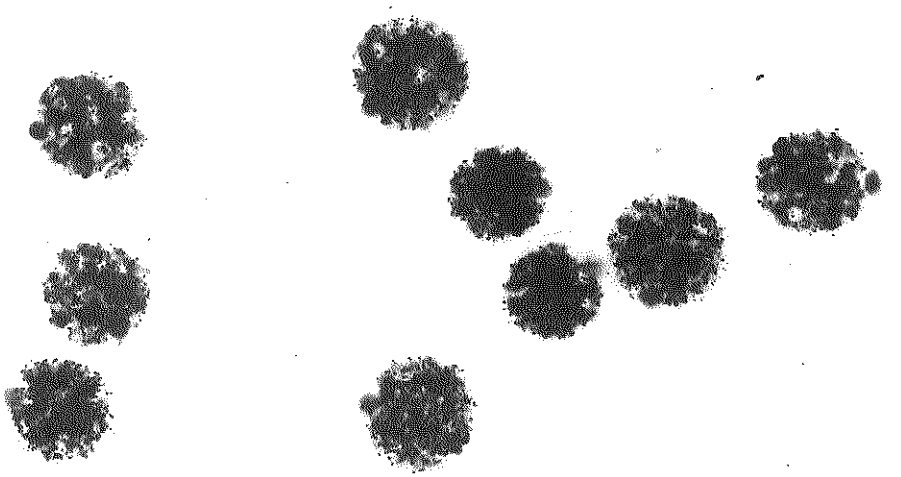


Figure 6 Radioautogram of isolated pachytene spermatocytes after incubation of the cells <u>in vitro</u> with ^{3}H-uridine (5 µCi/ml, specific radioactivity 5 Ci/mmol) for 2 h, showing grains over the nuclei. The cells were fixed with a mixture of absolute ethanol (3 parts) and glacial acetic acid (1 part) and were stained with Giemsa (X 1000).

Chapter 3

SEVERAL BIOCHEMICAL ASPECTS OF HORMONE EFFECTS ON GERMINAL CELLS

It is generally accepted that follitropin and testosterone have a sustaining effect on spermatogenesis in vivo (174). In order to study the molecular mechanism of action of these hormones on spermatogenesis it is essential to know which testicular cell types are the primary target of follitropin and testosterone (section 3.1).

We have studied a restricted part of spermatogenesis which was expected to be sensitive to hormones, namely the primary spermatocytes in the pachytene stage of the meiotic prophase (chapter 1). Recently much progress has been made in the biochemical analysis of DNA metabolism at the pachytene stage related to the crossing-over process, which is believed to be the mechanism for exchange of genetical information between the paired homologous chromosomes (74,75,114). Also, the pachytene spermatocytes have been found to incorporate precursors into protein and RNA (130) and it is conceivable that the newly-synthesized macromolecules play an important role. However, convincing evidence for effects of hormones on molecular processes in pachytene spermatocytes is not available.

Initially we have studied the activity of two enzymes and protein synthesis because it had been shown that marked changes could occur in these biochemical parameters in testicular tissue (section 3.2 and 3.3). Enzyme activities were estimated in total testicular tissue because it was known that the activity of different enzymes changed at successive periods of development of the testis or after changes in the hormonal environment (section 3.2). Such changes in activity might not only coincide, but also functionally correlate with the development of pachytene spermatocytes. Protein synthesis was estimated in total testicular tissue and isolated germinal

cells in an attempt to correlate hormone-induced changes in testicular protein synthesis with protein synthesis in pachytene spermatocytes (section 3.3).

The results of the experiments on enzyme activities and protein synthesis described in section 3.2 and 3.3 are discussed mainly to illustrate some of the problems involved in the search for hormonally regulated processes in germinal cells. On the basis of the data in sections 3.1-3.3 we propose a scheme for additional experiments (section 3.4).

3.1 Target cells for follitropin and testosterone in the testis

3.1.1 Sertoli cells as target cells for follitropin

Follitropin binds to plasma membrane receptors before it can exert stimulatory effects (157). In rat testicular tissue specific binding of follitropin has been detected in plasma membranes of tubular cells (121). With autoradiography Orth & Christensen (138) observed binding of ^{125}I-labelled follitropin to plasma membranes of Sertoli cells, but some binding of the labelled follitropin to plasma membranes of spermatogonia could not be excluded. After irradiation of rats *in utero*, the testes of these rats after birth contain apparently normal Sertoli cells but germinal cells are initially nearly absent (46,119). These Sertoli-cell-enriched testes contained the same number of follitropin receptors as normal testes, and follitropin stimulated the production of cAMP, the activity of cAMP-dependent protein kinase and the synthesis of proteins in such testes (46).

Sertoli cells have also been isolated and used for studies *in vitro* (51,171,191). In the presence of follitropin in the medium of Sertoli cell cultures several properties of these cells are stimulated such as the production of cAMP (32), the incorporation of ^{3}H-leucine into acid-insoluble cellular material (32) or proteins which are secreted (149), the pro-

duction and secretion of a specific protein called androgen binding protein (ABP) (51), the synthesis of oestradiol-17β from added testosterone (31) and the incorporation of ^{3}H-thymidine into nuclear DNA (63). Moreover, the morphology of cultured Sertoli cells is influenced by follitropin (186).

Early spermatocytes pass from the basal tubular compartment to the adluminal tubular compartment (34,154). A "blood-testis barrier" separates the two tubular compartments (34) and ^{125}I-labelled rat follitropin does not penetrate readily into the adluminal tubular compartment (162). Spermatogonia and preleptotene spermatocytes in the basal compartment of the tubuli are the only germinal cell types which could be influenced by follitropin directly if they would possess receptors for follitropin.

The above observations indicate that follitropin primarily interacts with Sertoli cells.

3.1.2 Sertoli cells as target cells for testosterone

Intracellular receptor proteins are responsible for the nuclear uptake and binding of androgens, which is an obligatory step in the mechanism of action of these steroids (86, 110). Androgen binding protein (ABP), produced and secreted by the Sertoli cells *in vivo* (66) and *in vitro* (51), is a protein which specifically binds androgens. This extracellular protein, however, is different from the intracellular androgen receptor which has been found in rat testicular tubules (69,132).

In the testicular tubules, a nuclear receptor for androgens has been demonstrated in Sertoli cells (133, appendix paper 1) and several other observations indicate that the Sertoli cell indeed is a target cell for androgens. Addition of testosterone to the medium of cultured Sertoli cells increased the production and secretion of ABP (103) and other proteins (149). Also, androgens have been found to influence *in vivo* the activity of ABP in testicular tissue under dif-

ferent experimental conditions (40,181,189). The results from these in vivo experiments, however, may be questioned because recent findings indicate that the presence of testosterone during homogenization of the tissue influences the estimation of tissue ABP levels (180).

A nuclear receptor for androgens, comparable to the receptor in Sertoli cells, appears to be absent from spermatocytes and spermatids (appendix paper 1). In contrast, other authors have reported a possible binding of androgens to male germinal cells (54,156,184). The results of these studies have been discussed in appendix paper 1, and it was concluded that there is no convincing evidence for the presence of cytoplasmic or nuclear receptors for androgens in germinal cells.

In mice, it has been shown that the locus of the gene which codes for a cytoplasmic androgen receptor is on the X chromosome (9,108). After a mutation in this locus male mice are androgen-resistant and show the syndrome of testicular feminization (Tfm) (108). In such male mice, spermatogenesis progresses up to the first meiotic division (108,137). Pachytene spermatocytes therefore seem to be formed in the absence of a response to androgens. During their further development the germinal cells probably cannot synthesize an androgen receptor because in normal spermatogenesis the X and Y chromosomes become inactive with respect to RNA synthesis during the meiotic prophase (129) and half of the germinal cells do not possess an X chromosome when they are haploid after the first meiotic division. These results strongly suggest that the development of germinal cells is not dependent on the presence of androgen receptors in the germinal cells. In addition it has been shown that male mice, made chimaeric for androgen-resistant (Tfm/Y) and normal (+/Y) genotype, produced offspring with the Tfm mutation (107). Androgen resistant germinal cells therefore can develop and form functional spermatozoa if some of the Sertoli cells present in the tubules are of the genotype +/Y and can respond to androgens.

Therefore, the available biochemical and genetical in-

formation strongly suggests that testosterone does not act directly on the germinal cells.

In conclusion, the observations described in section 3.1 are in favour of a model which involves a direct action of follitropin and testosterone on Sertoli cells but not on the germinal cells.

3.2 Enzyme activities in testicular tissue

3.2.1 Enzyme activities during testis development

During the development of the testis in immature rats, the first appearance of germinal cell types can be correlated with changes in the activity of enzymes in total testicular tissue (163). We have estimated in rat testicular tissue the activities of the lysosomal enzyme β-glucuronidase (EC 3.2.1.31) and the membrane-bound enzyme γ-glutamyltranspeptidase (γ-GTP, EC 2.3.2.2). The testicular activity of β-glucuronidase is mainly present in Sertoli cells and spermatogonia, and almost no activity of β-glucuronidase has been found in pachytene spermatocytes and spermatids (42,113). Histochemically, activity of γ-glutamyltranspeptidase can be demonstrated in the tubules, probably associated with Sertoli cells, but hardly in the germinal cells (71). This observation was recently confirmed by Lu & Steinberger (105), who found γ-glutamyltranspeptidase activity in isolated Sertoli cells, whereas only a very low activity was found in isolated germinal cells. It has been reported that the specific activity of these enzymes changed markedly in testes of immature rats between 10 and 40 days of age (71,113). We have estimated the specific activity and in addition the total activity per testis of the two enzymes during the development of the testis.

For these experiments testes were collected from 2-5 rats at different ages between 10 and 60 days of age. The testes were homogenized and enzyme activities were estimated and expressed as activity per mg of protein (specific activity) and as activity per testis. Also, testes were fixed in

Bouin's fixative and sections were made for histology, to estimate the time at which spermatogonia B, pachytene spermatocytes, and early spermatids appear in the testes.

As illustrated in Fig. 1, from 15-28 days of age the specific activity of β-glucuronidase decreased whereas the specific activity of γ-glutamyltranspeptidase increased sharply. These changes in specific activity occurred at the

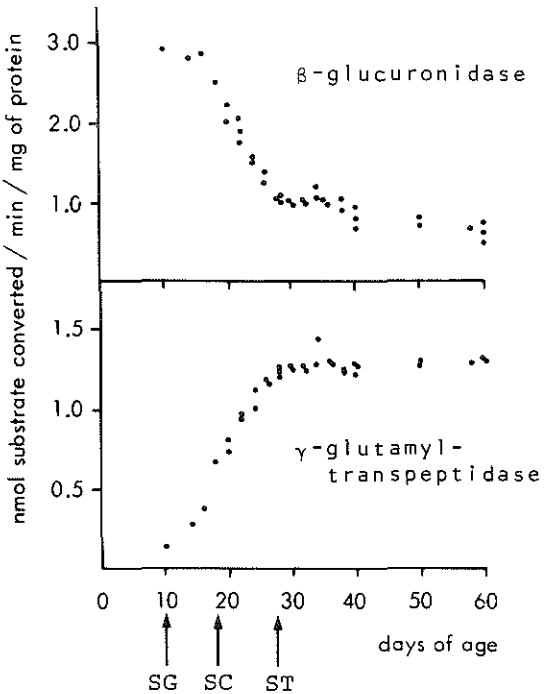


Figure 1 Specific enzyme activities in testicular tissue from rats of different ages. Testicular tissue was homogenized in 0.1 M Tris-HCl, pH 7.0 (20% homogenate w/v) and the activity in the homogenates of γ-glutamyltranspeptidase (γ-GTP) (177) and β-glucuronidase (113) was estimated. The dots in the figures represent the values obtained for 8 pooled testes (10-18 days of age) or the values obtained for 2 pooled testes from one rat (20-60 days of age). The time of appearance of germinal cell types is indicated with arrows (SG, spermatogonia B; SC, pachytene spermatocytes; ST, round spermatids).

time that the testes become populated with pachytene spermatocytes, after approximately 18 days of age. In 28 day-old rats the first spermatocytes give rise to early spermatids and after that time the specific activity of both enzymes did not further change. However, when the enzyme activities were expressed as total activity per testis (Fig. 2) the enzyme activities increased almost parallel with the testicular

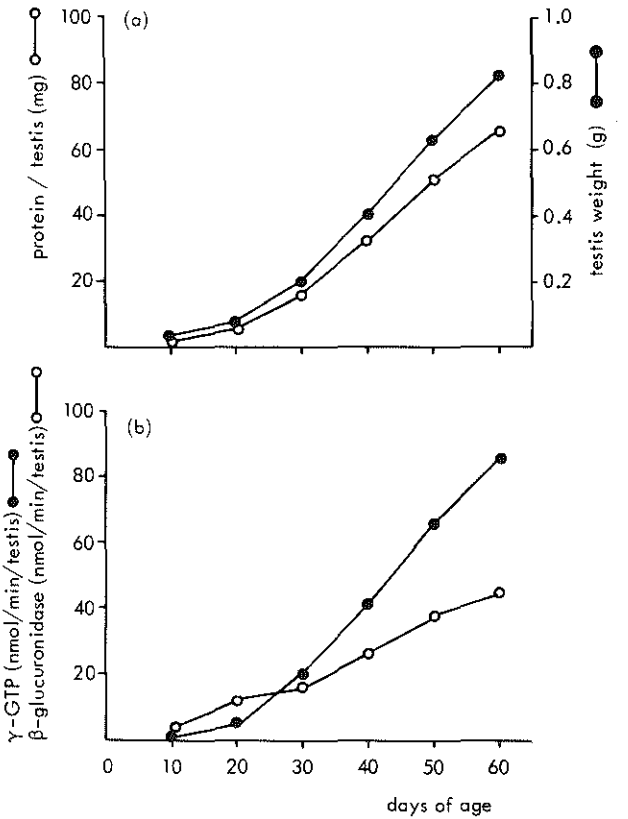


Figure 2 Total enzyme activities in testicular tissue and testicular weights from rats of different ages. In Fig. 2a the mean weight and protein content per testis is plotted as a function of age. In Fig. 2b the values for the activity of β-glucuronidase and γ-GTP presented in Fig. 1 were expressed as activity per testis. Mean results, obtained for rats of 10, 20, 30, 40, 50 and 60 days of age are presented.

weight during testicular development.

The specific enzyme activities (Fig.1) are influenced by changes of the cellular composition of the testis during development. The increase of the total enzyme activities per testis between 10 and 60 days of age (Fig.2) is apparently not specifically correlated with the first appearance in the testis of either pachytene spermatocytes or spermatids. From these results a functional correlation between a change in enzyme activities in Sertoli cells and the occurrence of a specific step in spermatogenesis can not easily be found, the more so because the results obtained for total testicular tissue could represent a heterogeneous population of Sertoli cells.

3.2.2 Effect of hormones on testicular enzyme activities

Several authors have tried to correlate changes in enzyme activities with hormonal influences on the testis. Elkington & Blackshaw (42,43) investigated effects of follitropin, lutropin and testosterone on the activity of β-glucuronidase in correlation with effects on tissue composition. In these studies changes in activity of the enzyme could be largely explained by changes in the population of testicular cell types. The authors concluded that the close correlation between changes in enzyme activity and cellular composition of the testis indicates that the synthesis of β-glucuronidase is not directly controlled by hormones but may reflect changes in cellular and cytoplasmic composition of the testis under different hormonal conditions (43). Similar conclusions most probably apply to studies on the hormonal control of acid phosphatase (42,43,113), acid proteinase (42,43), sorbitol dehydrogenase (127), uridine diphosphatase (196), and the isoenzymes of N-acetyl-β-glucosaminidase and β-galactosidase (112).

The testis-specific lactate dehydrogenase isoenzyme designated as LDH-X appears in the testis of the rat between 20 and 30 days of age (6). A recent study has shown that this isoenzyme is located mainly in pachytene spermatocytes and

spermatids (124). After hypophysectomy the isoenzyme disappears from the testis (42,44,194), but this can be prevented by administration of either follitropin and lutropin or testosterone (44,194). It has been speculated that hormones turn-on the gene responsible for the synthesis of the testis-specific subunit of LDH-X (194). A direct effect of hormones on the gene, however, has not been proven experimentally and this result could also be explained if it is assumed that hormones control the development of germinal cells through yet unknown mechanisms and that testicular LDH-X activity is dependent on the presence of pachytene spermatocytes and spermatids.

In conclusion, it appears that the study of a possible hormonal control of enzyme activities in total testicular tissue generally leads to results which cannot easily be interpreted due to changes in the cellular composition of the testis. Studies using the isolated cell types may therefore be more suited to estimate cellular enzyme activities and to define the enzymes which can serve as a marker for hormone effects on Sertoli cells and specific germinal cell types.

3.3 Effect of follitropin on protein synthesis

In this section observations are described on the short-term effect of follitropin on protein synthesis in testicular tissue.

Means & Hall (117) have reported that the incorporation *in vitro* of radioactively labelled amino acids into proteins of total testicular tissue from immature rats was stimulated after administration *in vivo* of follitropin. This stimulation was hormone-specific, dose-dependent and maximal (170% over control) 1 h after injection of the hormone. The response to follitropin was found in 20 day-old rats and was absent when the rats were more than 28 days old, but with adult animals stimulation of protein synthesis by follitropin was observed within 1 day after hypophysectomy (118). Means & Hall (118) suggested that follitropin stimulated protein synthesis in primary spermatocytes and possibly also in Sertoli cells and

Table I Effect of follitropin on the incorporation of ^{3}H-leucine into acid-precipitable material of testicular cells. 30 Day-old rats (2 days after hypophysectomy) were injected intraperitoneally with 0.9% NaCl or follitropin (ovine NIH-FSH, 1 µg/g body weight) and after 60 min the rats were killed. The testes were slightly teased and were then incubated for 45 min at 32°C with 5 µCi of ^{3}H-leucine (0.1 mM)/ml in PBS-buffer (pH 6.9) (33). Subsequently, cell suspensions were prepared from the pooled testes of the control group and the follitropin-treated group (6 rats each). Separation of the cells was performed by sedimentation at unit gravity at 5°C in a Ficoll gradient and fractions enriched in pachytene spermatocytes were collected (chapter 2). The amount of radioactivity incorporated into acid-precipitable material of total cell suspensions and of pachytene spermatocyte fractions was estimated essentially as described by Means and Hall (117). The amount of protein was estimated as described by Lowry et al. (104) and the results are expressed as dpm per mg of protein.

Fraction	Experiment number	10^{-3} x ^{3}H-leucine incorporated (acid-precipitable dpm/mg of protein)	
		0.9% NaCl	follitropin
Total cell suspension	1	19.1	22.2
	2	20.3	20.4
Pachytene spermatocyte fraction	1	27.3	30.6
	2	28.7	26.1

spermatogonia. We decided to use a cell separation technique to define whether protein synthesis is accelerated in isolated germinal cell types after administration in vivo of follitropin.

For the present experiments, rats 30 days of age were used 2 days after hypophysectomy. The rats were divided in two groups and either follitropin (1 μg/g body weight) or 0.9% NaCl was injected intraperitoneally. After 1 h the rats were killed and the testes from each group of rats were pooled. The testes were slightly teased and incubated with ^{3}H-leucine at 32°C for 45 min. Subsequently cell suspensions were prepared with trypsin, the cell suspensions were cooled to 5°C and separated by velocity sedimentation at unit gravity (chapter 2). Fractions enriched in pachytene spermatocytes were collected and the radioactivity incorporated into acid-precipitable material was estimated (Table I). It appeared that administration in vivo of follitropin had no effect on the incorporation of ^{3}H-leucine in either the total cell suspension applied to the sedimentation column or in the spermatocyte fractions obtained after separation.

However, it is possible that the conditions of these experiments did not allow detection of an effect of follitropin. We have therefore repeated one of the experiments as described by Means & Hall (117), using the same experimental conditions to estimate effects on the synthesis of proteins in total testicular tissue of 20 day-old rats (see legend to Fig. 3).

The results, presented in Fig. 3, illustrate that the incorporation of ^{3}H-leucine in follitropin-treated testicular tissue was not different from that in control tissue. In another experiment, no stimulation of protein synthesis was observed when the rats were injected with follitropin followed after 1 h by injection of ^{3}H-leucine in the tail vene and killing of the rats 30 min after the start of the labelling in vivo (Fig. 4). The rats used for this in vivo labelling experiment were either normal 20 day-old rats or prenatally irradiated 20 day-old rats, which have no active spermatogenesis and the testes of these irradiated rats are therefore

enriched in Sertoli cells (46).

From these results we concluded that in the present experiments follitropin, administered *in vivo*, probably did not stimulate protein synthesis in testicular tissue *in vivo* or *in vitro*. We have not measured the endogenous pool-size of amino acids, but changes in the size of this pool could have disturbed the detection of changes in the rate of protein synthesis. Means & Hall (117), however, did not observe changes in the transport of ^{14}C-aminoisobutyric acid which has a transport rate similar to that of the neutral amino acids. In contrast, Irusta & Wassermann (78) have re-

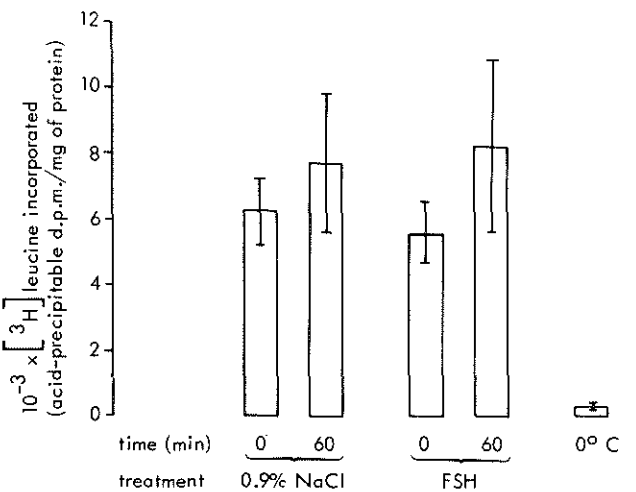


Figure 3 Effect of follitropin administration *in vivo* on the incorporation *in vitro* of ^{3}H-leucine in testicular tissue. From 20 day-old rats one testis was removed (0 min control). Subsequently the rats were injected with either 0.9% NaCl or follitropin and after 60 min the other testis was removed. The testes were incubated at 37°C for 30 min with 1 µCi of ^{3}H-leucine (0.1 mM)/ml in Krebs Ringer bicarbonate buffer (pH 7.4) in an atmosphere of 5% CO_2 in O_2 as described by Means & Hall (117). As a control for aspecific incorporation other testes were also incubated with ^{3}H-leucine at 0°C. Mean values (± S.D., n = 6) of the incorporation of ^{3}H-leucine into acid-precipitable material (117) are presented.

ported an increased transport of ^{14}C-aminoisobutyric acid in testicular tissue *in vitro* 4 h after follitropin administration *in vivo* to 22 day-old rats. Davies et al. (25,26,90) have used prepubertal and hypophysectomized adult mice for experiments similar to the experiments of Means & Hall (117). They observed that the incorporation of ^{3}H-lysine into testicular protein *in vivo* was increased with a maximal response 8-16 h after subcutaneous injection of follitropin. The possibility that follitropin enhanced the uptake of ^{3}H-amino acids in the testes of these mice, however, has not been experimentally excluded (25,90) and therefore it is not certain whether follitropin stimulated protein synthesis.

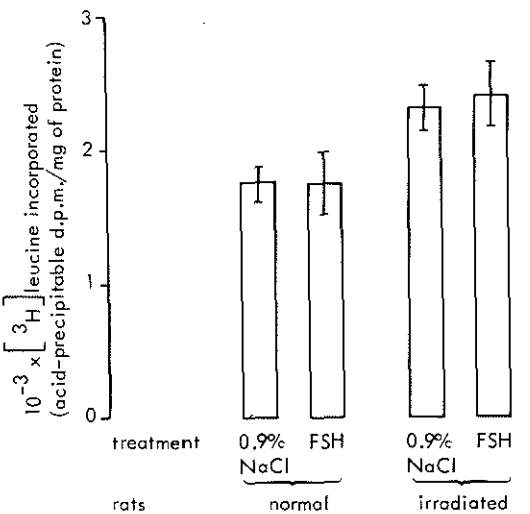


Figure 4 Effect of follitropin administration *in vivo* on the incorporation *in vivo* of ^{3}H-leucine in testicular tissue. 20 Day-old rats (either normal or prenatally irradiated) were injected with either 0.9% NaCl or follitropin. After 1 h ^{3}H-leucine (5 µCi) was injected into the tail vene and 30 min later the rats were killed and the testes were collected. Mean values (± S.D., n = 8) of the incorporation of ^{3}H-leucine into acid-precipitable material (117) are presented.

Several observations, described in section 3.1, strongly suggest that follitropin primarily exerts a stimulatory effect on biochemical processes in Sertoli cells. The mechanism of action of follitropin in the testicular tissue has been studied and appeared to involve activation of adenylate cyclase and cAMP-dependent protein kinase (120). The ultimate effect of follitropin on protein synthesis in testicular tissue as observed by Means & Hall (117), is located most probably in the Sertoli cells (46).

In addition, follitropin appears to influence also the germinal cells in immature rats. For example, it has been shown that degeneration of type A spermatogonia which occurs in normal 16 day-old rats could be largely prevented by injection of follitropin (76). Moreover, selective withdrawal of follitropin with an antiserum to rat follitropin results in a disturbance of spermatogenesis in immature rats (109).

At this moment, it is not possible, however, to associate the biochemical effects in testicular tissue observed after follitropin administration with the presence of a mechanism which could be responsible for effects on the development of germinal cells.

3.4 A scheme for further work

Sertoli cells appear to be target cells for follitropin and testosterone, whereas follitropin does probably not act directly on germinal cells and receptor proteins for androgens are not present in germinal cells (section 3.1). It is therefore likely that follitropin and testosterone do not influence the development of spermatocytes via direct interactions with the germinal cells. As a working hypothesis, it can be considered that, after hormonal stimulation, changes in properties of Sertoli cells may influence molecular processes in the germinal cells.

It has been discussed (section 3.2) that experiments with total testicular tissue cannot easily distinguish between properties of germinal cells and other testicular

cell types. The study of isolated Sertoli cells might provide information on cell-specific factors which are hormone-dependent. Such factors could interact with germinal cells, a suggestion which may be examined if a technique to study the properties of isolated germinal cells is available.

In experiments described in chapter 4 we have used isolated spermatocytes to obtain information about RNA synthetic properties of spermatocytes. Subsequently an attempt can be made to study whether the action of hormones on the germinal epithelium influences RNA synthesis in spermatocytes. From the information in section 3.1 it is evident that the germinal cells should be under the influence of Sertoli cells during the exposure to different hormonal conditions. Therefore, it was decided to examine the incorporation of radioactively labelled uridine into RNA in spermatocytes from normal rats and from hypophysectomized rats. The hypophysectomy will cause a fall in the level of hormones and changes in RNA synthesis in spermatocytes could occur after hypophysectomy if RNA synthesis in spermatocytes is dependent on the presence of hormones.

If, indeed, properties of the germinal cells (e.g. RNA synthesis) are found to be different for cells isolated from normal and hypophysectomized rats, these properties could be used to examine *in vitro* the influence exerted by Sertoli cells and hormones on RNA synthesis and other properties of spermatocytes.

Chapter 4

RNA SYNTHESIS IN PACHYTENE SPERMATOCYTES

At the start of our studies it was known from experiments with radioautographic and biochemical techniques that pachytene spermatocytes of different mammalian species incorporate ^{3}H-uridine into RNA (83,98,130,166,187). Most of the previous biochemical studies were performed with total testicular tissue (53,134) or seminiferous tubules (165,166). Information on the incorporation of uridine into RNA in isolated germinal cells, however, was not available. Such information might be important in studying possible effects of hormones on RNA synthesis in germinal cells (chapter 5) and we have therefore studied with biochemical techniques the incorporation of ^{3}H-uridine into RNA in isolated pachytene spermatocytes (section 4.1 and 4.2, appendix paper 2). In order to demonstrate the specificity of the pattern of RNA synthesis in spermatocytes, also the RNA in Sertoli cells, peritubular cells and spermatids was radioactively labelled and characterized (section 4.3, appendix paper 2). Finally, in section 4.4, observations and suggestions of other authors which relate to the present findings on the synthesis of RNA in spermatocytes are discussed.

4.1 Incorporation of ^{3}H-uridine into RNA of isolated spermatocytes

RNA synthesis in spermatocytes was examined using the incorporation of radioactively labelled uridine. The spermatocytes can incorporate ^{3}H-uridine under different incubation conditions, viz. 1. *in vivo*, 2. *in vitro* when present in isolated tubular fragments and 3. *in vitro* as isolated cells. Methods 1 and 2 are attractive since the spermatocytes are in contact with Sertoli cells and are probably not damaged when the labelled precursor is incorporated. A major disadvantage

of these methods, however, is that the labelling conditions are not well defined and a quantitative assay of the incorporation of labelled uridine cannot be performed. Also, the synthesis of labile RNA species cannot be studied since the labile RNA species are partly degraded during the isolation of the cells after labelling.

The labelling conditions can be better controlled when isolated spermatocytes are incubated with ^{3}H-uridine. At a low uridine concentration in the medium the cellular uptake and incorporation of uridine occurred for at least 4 h after isolation of the cells (Fig. 1). The amount of uridine which is taken up by the cells and incorporated into RNA increased with increasing amounts of uridine in the medium until saturation was obtained at uridine concentrations above 60 µM

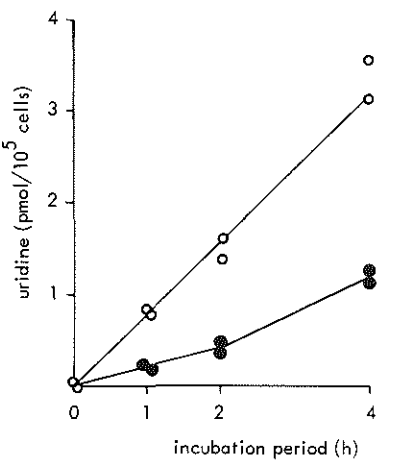


Figure 1 Uptake and incorporation of uridine by isolated spermatocytes. Isolated spermatocytes were incubated with 0.45 µM ^{3}H-uridine (20 µCi/ml). At different time intervals after addition of the label, the uptake and incorporation of ^{3}H-uridine was estimated in cells from duplo incubations and expressed as pmol uridine/10^5 cells.

o——o uptake

•——• incorporation

(Fig. 2). The relative rate of ^{3}H-uridine incorporation, defined as the ratio of incorporated radioactivity to the total uptake of radioactivity, was hardly affected by the concentration of uridine in the medium (Fig. 2). From the data in Fig. 2 it was calculated that the incorporation of extracellular uridine into RNA of isolated spermatocytes was maximally 30 pmol/2 h/µg of DNA. The base composition of rapidly labelled RNA in testicular tissue has been estimated (134) and this RNA contains about 25% of uridylic acid. The rate of RNA synthesis, calculated from these data, was about 30 ng of RNA synthesized/2 h/µg of DNA. This is probably an underestimation of the actual rate of RNA synthesis since turnover of RNA, endogenous pool size and label equilibration have not been taken into account. The isolated cells contain about 1.6 µg of RNA/µg of DNA and therefore it appears that at least 2% of total RNA was newly synthesized every 2 h.

The incorporation of ^{3}H-uridine was inhibited when actinomycin D, an inhibitor of RNA synthesis (143), was added to the incubation medium at the same time as the ^{3}H-uridine (Fig. 3). The inhibition was almost complete and very rapid at actinomycin D concentrations above 8 µg/ml. The results in Fig. 3 indicate that the ratio of incorporated radioactivity to the total uptake of radioactivity, which decreases when the actinomycin D concentration is increased, is a useful criterion to express changes in the relative rate of ^{3}H-uridine incorporation.

In appendix paper 2 it is reported that the incorporation of ^{3}H-uridine into RNA of isolated spermatocytes was decreased after 20 h of preincubation. In later experiments, however, this decrease was frequently not observed and the combined results show therefore a large variation (incorporation of ^{3}H-uridine after 20 h of preincubation, expressed as a percentage of the incorporation in freshly isolated cells, was 68 $\pm$ 57, $\pm$ S.D., n = 6). However, the relative rate of ^{3}H-uridine incorporation was not changed after 20 h of preincubation (ratio of incorporated radioactivity to total uptake of radioactivity was 0.39 $\pm$ 0.05 in freshly isolated

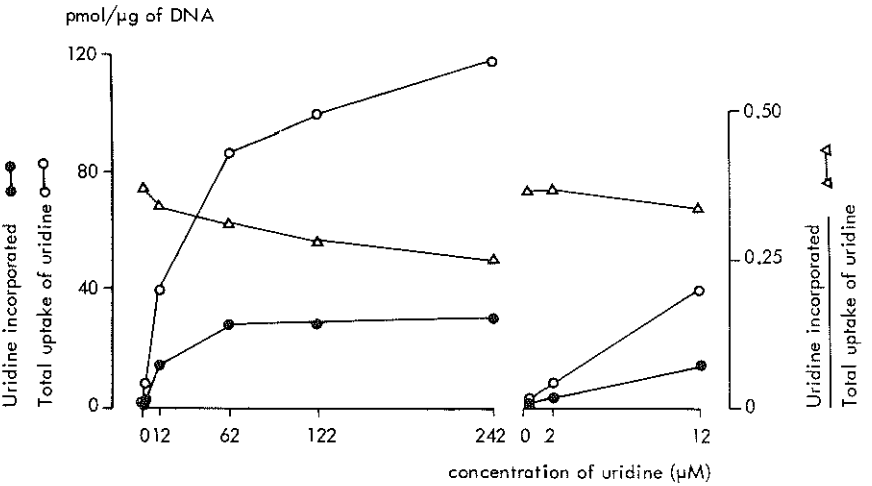


Figure 2 Effect of uridine concentration on uptake and incorporation of ^{3}H-uridine. Isolated spermatocytes were incubated for 2 h with different concentrations of ^{3}H-uridine (10 µCi/ml). Uptake and incorporation of uridine were expressed as pmol/µg of DNA.

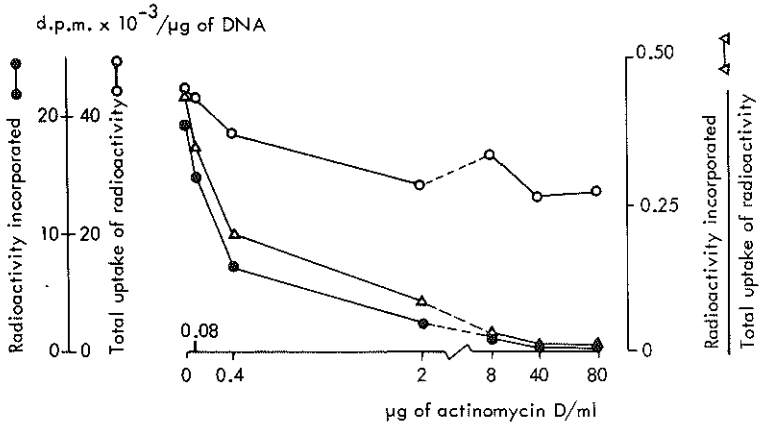


Figure 3 Effect of actinomycin D on uptake and incorporation of ^{3}H-uridine. Isolated spermatocytes were incubated for 2 h with ^{3}H-uridine (10 µCi/ml, specific racioactivity 30 Ci/mmol) in the presence of different concentrations of actinomycin D. Uptake and incorporation of radioactivity were expressed as d.p.m./µg of DNA.

spermatocytes and 0.38 ± 0.04 in spermatocytes after 20 h of preincubation, ± S.D., n = 6).

In conclusion, it appears from the observations described in this section as well as from results described in appendix paper 2 that isolated spermatocytes can be used for at least 8 hours after isolation to study uridine incorporation in these cells. When the isolated cells are used information can be obtained on changes in the relative rate of ^{3}H-uridine incorporation.

4.2 Contribution to total RNA synthesis of heterogeneous RNA, rRNA and tRNA

The results obtained with isolated spermatocytes show that the spermatocytes synthesize rapidly labelled RNA with a heterogeneous electrophoretic mobility, designated as heterogeneous RNA (appendix paper 2). In radioautograms accumulation of label in the nucleus of spermatocytes is seen after incubation with ^{3}H-uridine for 2 h and the heterogeneous RNA therefore is probably mainly nuclear RNA. In somatic cells the major product of transcription is different from rRNA and tRNA and this RNA is called heterogeneous nuclear RNA (hnRNA, Fig. 4) (190). HnRNA is considerably larger than mRNA and most of the hnRNA does not leave the nucleus but is rapidly broken down (190). Possibly hnRNA contains precursors for mRNA molecules (96). A large part of the hnRNA, however, may have a different function (193). Whether or not the heterogeneous RNA in spermatocytes is functionally related to hnRNA in somatic cells has not been shown. Most of the heterogeneous RNA in spermatocytes is probably labile, with a rapid turnover comparable to that of hnRNA in somatic cells (158). A small part of the heterogeneous RNA in spermatocytes, however, can belong to a class of stable RNA (appendix paper 2, section 4.4).

Distinct peaks of labelled rRNA were hardly observed when spermatocytes were labelled with ^{3}H-uridine for less than 2 h. Some rRNA has been formed during short labelling

periods, but in the electrophoretic profile this RNA was overshadowed by the labelled heterogeneous RNA. Labelled rRNA components (Fig. 4) became visible after labelling with ^{3}H-uridine for longer time periods or when the labelled heterogeneous RNA was processed during an incubation with actinomycin D (appendix paper 2).

In the pachytene spermatocytes ^{3}H-uridine is incorporated also into 4S tRNA which can be shown when spermatocytes are incubated in the presence of L-(methyl-^{14}C)methionine. The methyl-^{14}C group is used for methylation of different RNA fractions but mainly for methylation of tRNA (7). In spermatocytes we observed incorporation of methyl-^{14}C as well as ^{3}H-uridine into a single peak which was therefore believed to contain the 4S tRNA (Fig. 5).

In summary, the isolated pachytene spermatocytes synthesize mainly labile heterogeneous RNA, whereas the incorporation of ^{3}H-uridine into rRNA components occurs much slower. Synthesis of tRNA has also been observed. Similar observations were made when the RNA of the spermatocytes was labelled with ^{3}H-uridine <u>in vivo</u> (appendix paper 2).

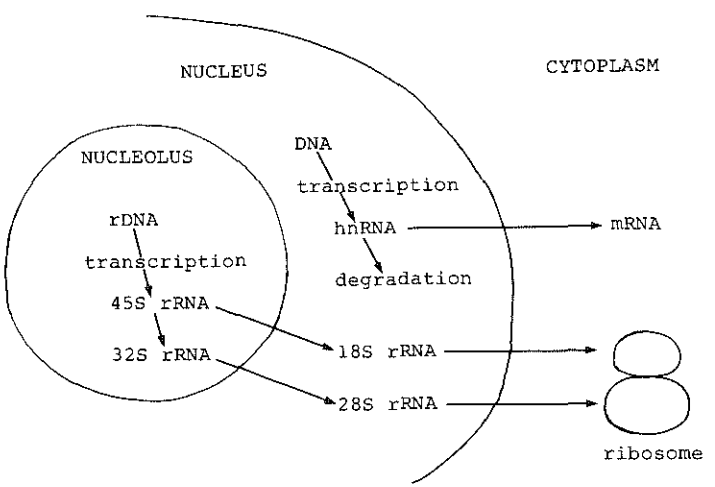


Figure 4 Schematic presentation of the synthesis and processing of RNA.

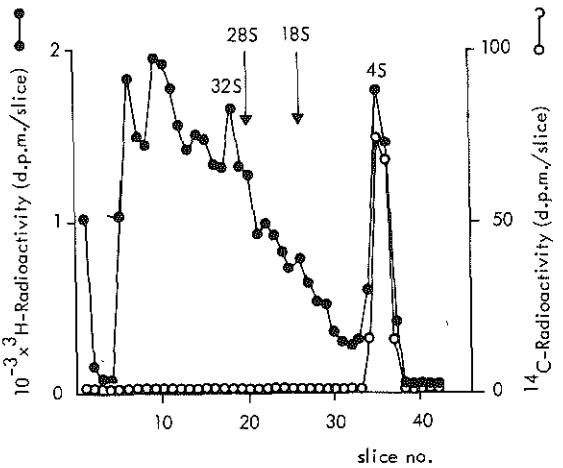


Figure 5 Incorporation of ^{3}H-uridine and methyl-^{14}C into RNA of isolated spermatocytes. Isolated spermatocytes were incubated for 4 h with ^{3}H-uridine (20 µCi/ml, specific radioactivity 30 Ci/mmol) and L-(methyl-^{14}C)methionine (20 µCi/ml, specific radioactivity 51 mCi/mmol). RNA was extracted and separated by electrophoresis and ^{3}H- and ^{14}C-radioactivity was estimated in the gel slices.

4.3 Cellular specificity of the incorporation of ^{3}H-uridine into RNA of spermatocytes

Electrophoretic profiles of RNA from spermatocytes, Sertoli cells and peritubular cells, isolated from rat testes and labelled during 8 h of incubation of the cells with ^{3}H-uridine, are compared in Fig. 6. Whereas the A_{260} profiles, showing 28S and 18S rRNA peaks, were similar for the three cell types, two main differences were observed in the profiles of labelled RNA. Firstly, the specific radioactivity of the rRNA components was markedly different resulting in a small contribution of rRNA components to the profiles of labelled RNA in spermatocytes as compared to that in Sertoli cells and peritubular cells. The contribution of RNA species with a heterogeneous electrophoretic mobility to the profiles of labelled RNA was relatively

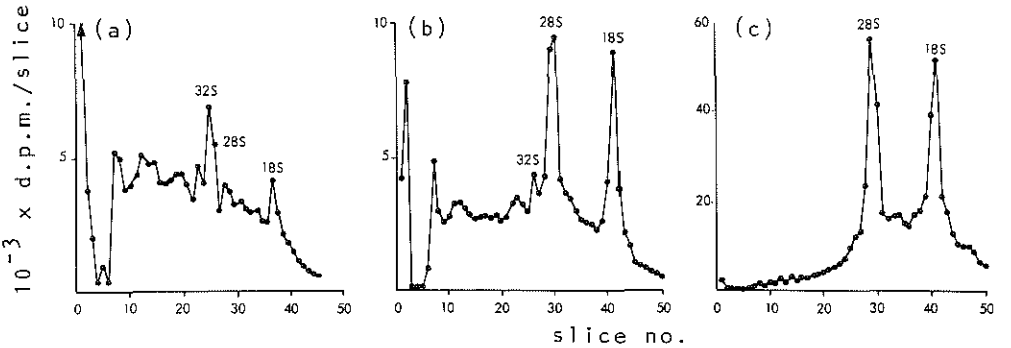


Figure 6 RNA synthesis in isolated spermatocytes, Sertoli cells and peritubular cells. Spermatocytes were preincubated for 2 h, Sertoli cells and peritubular cells for 5 days. The cells were incubated for 8 h with ^{3}H-uridine (10 µCi/ml, specific radioactivity 29 Ci/mmol).
a) spermatocytes; b) Sertoli cells; c) peritubular cells.

high in spermatocytes. The second difference is the presence of a 32S component in the profile of labelled RNA from spermatocytes which was small or absent in the profiles of labelled RNA from Sertoli cells and peritubular cells. Also in spermatocytes, but not in Sertoli cells and peritubular cells, less radioactivity was incorporated into 28S rRNA than into 18S rRNA after 8 h of incubation with ^{3}H-uridine (Fig. 6). This observation suggested that the formation of 28S rRNA was retarded in the spermatocytes, whereas the 45S precursor was cleaved without a marked delay since 18S rRNA had been formed. It can be postulated therefore that the 32S RNA component in spermatocytes is the ribosomal RNA precursor of 28S rRNA (appendix paper 2) (Fig. 4).

The labelled 32S rRNA precursor is probably also cleaved very slowly in other tubular cells than pachytene spermatocytes as indicated by the following observation. After 8 h of incubation of tubular fragments in the presence of ^{3}H-uridine both a labelled 32S and a 28S component were observed in the electrophoretic profile of RNA (Fig. 5 in chapter 2, p.28). The ratio

of the radioactivity in the 32S peak to the radioactivity in the 28S peak (32S/28S), calculated from profiles of labelled RNA from tubular fragments, could give an indication for the presence and activity of cell types in these tubular fragments which synthesize the labelled 32S component. The ratio 32S/28S was estimated in tubular fragments as a function of age of the rats (Fig. 7). This ratio increased between 5 and 25 days of age and was not precisely correlated with the appearance of pachytene spermatocytes in rats after 18 days of age. It is likely therefore that cell types, different from pachytene spermatocytes, also contributed to the presence of labelled 32S RNA in tubular fragments after incubation with ^{3}H-uridine for 8 h. This synthesis of 32S RNA in young rats could reflect the activity of early germinal cell types but not of Sertoli cells and peritubular cells, because in these somatic cells the ratio 32S/28S was very low (Fig. 6).

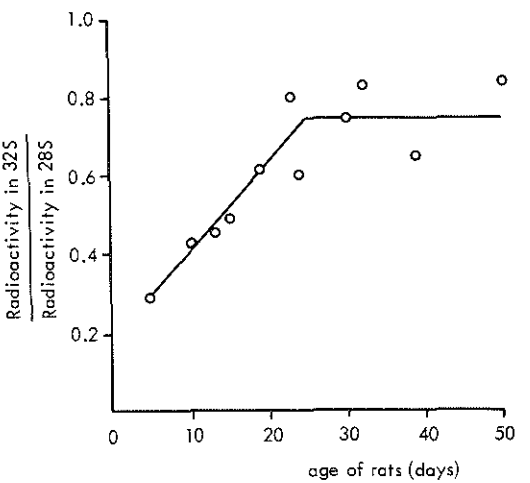


Figure 7 ^{3}H-labelled 32S rRNA in tubules. Tubular fragments from rats of different ages were incubated for 8 h with ^{3}H-uridine (25 µCi/ml, specific radioactivity 29 Ci/mmol). RNA was extracted and was separated by electrophoresis. The ratio of radioactivity present in the 32S peak to that in the 28S peak is presented as a function of age.

Synthesis of 28S and 18S rRNA has been observed in cell preparations enriched in round spermatids, whereas almost no labelled 32S component was present (appendix paper 2). Kierszenbaum & Tres (85) studied the mouse spermatid genome at the electron microscopic level and did not detect structural or autoradiographic evidence for the presence of nucleoli in these spermatids. This observation could reflect that the spermatids do not synthesize rRNA and that the synthesis of rRNA, which was observed in our experiments in the spermatid preparations was the result of the presence of contaminating cells. Using radioautography, it was found that the spermatid preparations contained approx. 3% of non-germinal cells, which were much more active with respect to ^{3}H-uridine incorporation than round spermatids. The presence of such cells in the spermatid preparations could result in the erroneous conclusion that rRNA is synthesized in round spermatids. A similar contamination could reduce the significance of the observations made on rRNA synthesis in isolated spermatocytes. The number of RNA synthesizing non-germinal cell types in cell preparations enriched in pachytene spermatocytes, estimated from radioautograms, was 2.5% $\pm$ 1.8% (mean $\pm$ S.D., n = 5). The number of grains per nucleus in these non-germinal cells was lower than that per nucleus of pachytene spermatocytes, but these non-germinal cells may still contribute to the synthesis of 28S and 18S rRNA in spermatocyte preparations. This contribution must be quite small, however, since after 8 h of incubation with ^{3}H-uridine only a small amount of labelled 28S rRNA, relative to the amount of labelled 32S and 18S rRNA, was observed in the spermatocyte preparations (Fig. 6 and appendix paper 2).

Recently Geremia et al. (55) observed the presence of labelled 28S and 18S rRNA in the post-mitochondrial supernatant of preparations of pachytene spermatocytes and of round spermatids. These cell preparations were obtained from mouse seminiferous tubules, labelled with ^{3}H-uridine *in vitro* for 3 h, and were virtually free of contaminating somatic cells.

In summary, it appears that the pattern of incorporation of ^{3}H-uridine into RNA in spermatocytes is different from that in somatic cells. During the pachytene stage total rRNA and particularly 28S rRNA appears to be synthesized at a relatively low rate.

4.4 Synthesis of heterogeneous RNA and rRNA in pachytene spermatocytes

4.4.1 Stability and possible function of the heterogeneous RNA

Several authors have published observations on the synthesis of RNA in testicular tissue or seminiferous tubules from mammals. An RNA fraction which has been called rapidly-labelled nuclear RNA or hnRNA was suggested to be synthesized mainly in the spermatocytes (83,134,165,166). The heterogeneous RNA which was found to be synthesized in pachytene spermatocytes in the present experiments (appendix paper 2, section 4.2) represents probably a similar RNA fraction.

There is no agreement about the stability of this RNA fraction. Söderström (165,166) suggested that pachytene spermatocytes synthesize heterogeneous nuclear RNA which is stable for at least 36 hours. In contrast, the results of the present experiments indicate that most of the heterogeneous RNA in pachytene spermatocytes is labile (appendix paper 2). Also, Kierszenbaum & Tres (82,83,84) observed with autoradiographic techniques that a small part of the incorporated radioactivity from ^{3}H-uridine persisted in the nuclei of pachytene spermatocytes for several days but the authors suggest that this radioactivity represents degraded heterogeneous RNA.

The stability of the meiotic heterogeneous RNA is of interest with respect to a model, proposed by Monesi (130), Söderström (165) and Geremia (56), which involves that mRNA molecules or precursors of mRNA, synthesized in spermatocytes, are stable and are translated in late spermatids. This model could explain why in late spermatids active protein synthesis

can take place in the absence of transcription (130). It is difficult to prove biochemically that protein synthesis in spermatids is dependent on stable mRNA from spermatocytes, since it has been observed, for mammalian species, that the haploid genome of early round spermatids is transcribed (55, 56,85,131). An indication for translation of mRNA from spermatocytes in haploid spermatids has been obtained in a study on the mRNA for protamine in the developing trout testis (77). Protamine mRNA could be extracted from immature testes, which have not yet started to synthesize protamine. In spermatocytes, protamine mRNA was present in the cell sap but not in polysomes and only in testes at the spermatid stage protamine mRNA was bound to polysomes and translated (77).

The function of the heterogeneous RNA synthesized in pachytene spermatocytes is not clear, although it may be involved in the generation of mRNA, needed for housekeeping in the spermatocytes, and the formation of stable mRNA, needed for translation after meiosis. In mouse pachytene spermatocytes the heterogeneous RNA is probably synthesized on loops of chromosomes which resemble the lampbrush structures of the chromosomes in amphibian oocytes (84). From such observations, it has been suggested that in rat pachytene spermatocytes most genes are being transcribed (165). The presence of the lampbrush configuration in oocytes, however, does not seem to imply that an unusual large part of the genome is transcribed (24,92,168). Also, spermatocytes apparently do not synthesize an exceptional amount of heterogeneous RNA in spite of the formation of loop structures as shown by Geremia (56) who observed that the incorporation of ^{3}H-uridine, if measured under saturation conditions and expressed per amount of DNA, in spermatocytes is quantitatively the same as that in spermatids.

For oocytes, it has been suggested that transcription generates and maintains the loops of lampbrush chromosomes so that in the loops alterations of the deoxyribonucleoprotein fiber can take place which reprogram the chromosome for development (92). Similarly, it can be suggested that an important

function of the synthesis of heterogeneous RNA during the pachytene stage in spermatocytes is not related to the generation of a limited number of mRNA molecules but that the transcription process keeps the chromatin in a relatively unfolded state so that changes in the chromatin can occur which are needed for further development. In this context, it is of interest that synthesis of histones occurs during the pachytene stage (57, 60).

In summary, it appears likely that the heterogeneous RNA in pachytene spermatocytes is a labile RNA fraction which resembles hnRNA of somatic cells. A small part of the heterogeneous RNA in spermatocytes may serve as precursor of mRNA molecules which are translated in the spermatocytes during meiosis as well as after meiosis in the spermatids. The function of most of the heterogeneous RNA, however, is unknown.

4.4.2 Regulation of the synthesis of rRNA

With respect to the synthesis of rRNA in spermatocytes of mammals, the results presented in the literature (53,83,134, 165) and in sections 4.2 and 4.3 indicate a low relative rate of transcription of the rDNA. Furthermore, a labelled 32S RNA component and labelled 18S rRNA appear to be present in spermatocytes after 8 h of incubation with ^{3}H-uridine, whereas only a small amount of labelled 28S rRNA was formed (Fig. 6), which suggests that the cleavage of 32S rRNA was retarded. The function of such a slow synthesis and processing of the rRNA precursors in spermatocytes is unknown.

Some data in the literature indicate that rRNA synthesis can be specifically inhibited. For example, during the terminal step of oogenesis in Xenopus laevis an inhibitor of total rRNA synthesis is apparently formed in the oocyte (22,164). In Sciara salivary glands rRNA synthesis is probably under the inhibitory control of moulting hormone (29). Other observations reflect that the cleavage of rRNA precursors can be influenced. Alterations of the cleavage of the 45S rRNA

precursor have been observed (176,178,192), but also several systems are described in which the formation of 28S rRNA can be specifically influenced. For example, it has been shown that in rooster liver oestradiol can stimulate the processing of the 32S rRNA precursor to 28S rRNA (4). In oocytes of Xenopus laevis the processing of a nuclear 30S rRNA precursor to 28S rRNA is restricted when the follicles are not stimulated with human chorionic gonadotropin (2,67).

In summary, several observations on the synthesis of rRNA in non-testicular systems indicate that the formation of total rRNA and particularly the formation of 28S rRNA can be influenced by hormones or other regulatory mechanisms. The results obtained on rRNA synthesis in spermatocytes, indicating a relative suppressed rate of rRNA synthesis and a partial restriction of the processing, might reflect that similar regulatory mechanisms operate in these germinal cells.

Chapter 5

POSSIBLE EFFECTS OF HORMONES AND FACTORS FROM SERTOLI CELLS ON RNA SYNTHESIS IN PACHYTENE SPERMATOCYTES

From the results described in chapter 4 and in appendix paper 2 it appears that isolated pachytene spermatocytes can be used to estimate the relative rate of ^{3}H-uridine incorporation and to analyse the synthesis and processing of heterogeneous RNA and rRNA. Using this knowledge we have tried to study whether the pattern of the incorporation of uridine into RNA of spermatocytes could be influenced by the action of hormones or factors from Sertoli cells.

The development of the germinal cells at the pachytene stage is most probably dependent on the combined action of hormones and Sertoli cells (chapter 3). The effect of hormones on the incorporation of radioactively labelled RNA precursors has been studied by others in many different biological systems. This incorporation is influenced by steroid hormones (5,73,87,111,195) or protein hormones (52,116,146,147) through effects on different processes such as uptake and phosphorylation of nucleosides, incorporation of labelled precursors into different RNA fractions and further processing and transport of these RNA fractions. It might be suggested that follitropin and testosterone can influence in the presence of the Sertoli cell the incorporation of uridine into RNA in pachytene spermatocytes in a similar way. Therefore we have investigated the incorporation of radioactively labelled uridine into RNA under the following conditions: 1) in spermatocytes isolated from normal rats versus in spermatocytes isolated from hypophysectomized rats (section 5.1); 2) in spermatocytes at different time intervals after isolation versus in spermatocytes which were maintained *in vitro* in fragments of seminiferous tubules (section 5.2.1). For these tubular fragments also the effect of hormones was estimated (section 5.2.2); 3) in spermatocytes exposed to the medium of cultured Sertoli cells (section 5.2.3).

5.1 Effect of hypophysectomy on ^{3}H-uridine incorporation in pachytene spermatocytes

Pachytene spermatocytes were isolated from 26-35 day-old rats either directly or 2-4 days after hypophysectomy (appendix paper 3). After removal of the pituitary gland from immature rats the development of the testis is disturbed very rapidly as reflected by a decreased testis weight. After hypophysectomy also an increased number of degenerating spermatocytes was observed in the seminiferous tubules (appendix paper 3) and the composition of the spermatocyte preparations obtained with velocity sedimentation appeared to be changed. We therefore made corrections for the differences in composition of the spermatocyte preparations in order to obtain an impression about the true effect of hypophysectomy on the amount of ^{3}H-uridine incorporated into RNA of spermatocytes (appendix paper 3).

The incorporation of ^{3}H-uridine into RNA of spermatocytes isolated from hypophysectomized immature rats was decreased (approximately 50%). Most likely this decreased incorporation was not the result of a change in the relative rate of ^{3}H-uridine incorporation in the spermatocytes (appendix paper 3). This may reflect either that the uptake of ^{3}H-uridine was decreased in all isolated cells or that a part of the spermatocyte population was affected more severely. The results of radioautographic experiments (appendix paper 3) revealed that spermatocytes which apparently did not incorporate ^{3}H-uridine were more frequently (up to 25%) present in the cell preparations obtained from the hypophysectomized rats, whereas the uptake and incorporation of ^{3}H-uridine by the active spermatocytes were not or only slightly decreased. The morphology of the nuclei of these inactive spermatocytes, in Giemsa-stained preparations, was similar to that of spermatocytes which were incubated in the presence of actinomycin D, at a concentration which completely inhibits RNA synthesis. In both situations the chromosomes did not show diffuse margins but were condensed. This condensation of the chromatin can result from a

retraction of the loop structures which occurs when transcription is inhibited (8,148). Furthermore, in adult hypophysectomized rats, the nuclei of degenerating pachytene spermatocytes contained many patches of clumped chromatin (155). These observations indicate that the spermatocytes which did not incorporate ^{3}H-uridine are degenerated cells in which the structure of the chromatin has been changed.

Electrophoretic profiles of labelled RNA of spermatocytes from intact and hypophysectomized rats were compared after incubation of isolated cells or tubular fragments with ^{3}H- or ^{14}C-uridine for different time periods. An example of these experiments is presented in Fig. 1, whereas another example is presented in appendix paper 3. After 2 h of incubation of isolated spermatocytes with the labelled precursor ^{3}H-uridine was incorporated mainly into heterogeneous RNA and only small labelled rRNA peaks were present (Fig. 1). After 8 h of labelling of spermatocytes in tubular fragments labelled peaks of presumptive 32S, 28S and 18S rRNA were clearly present (appendix paper 3). Hypophysectomy did not result in significant changes in these electrophoretic profiles of labelled RNA (Fig. 1 and appendix paper 3) and therefore the relative contribution of the synthesis of heterogeneous RNA and rRNA to total RNA synthesis as well as the processing of these RNA species appeared unchanged.

In summary, the results of these experiments indicate that 2-4 days after hypophysectomy many pachytene spermatocytes can be isolated from the testes which incorporate radioactively labelled uridine apparently in a normal way. The activity of the total preparation of cells isolated from testes of hypophysectomized rats, however, was decreased, which can be partly explained by the observation that an increased percentage of the isolated spermatocytes did not incorporate ^{3}H-uridine. In addition it is possible that after hypophysectomy the uptake of uridine in the isolated cells was slightly decreased.

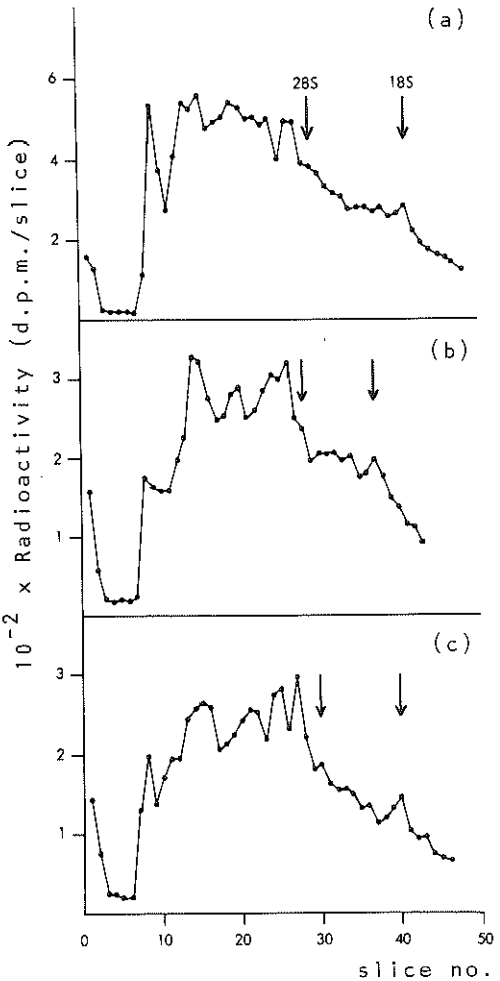


Figure 1 Electrophoretic profiles of ^{3}H-uridine incorporation into RNA of isolated spermatocytes. Spermatocytes were isolated from 25-29 day-old rats (intact, or 48 and 96 h after hypophysectomy). The isolated spermatocytes were incubated for 2 h in the presence of ^{3}H-uridine (50 µCi/ml, specific radioactivity 30 Ci/mmol). RNA was extracted from the spermatocytes and separated by gel electrophoresis. The position of 28S and 18S rRNA is indicated.

a) spermatocytes from intact rats

b) spermatocytes from rats 48 h after hypophysectomy

c) spermatocytes from rats 96 h after hypophysectomy

5.2 Effect *in vitro* of Sertoli cells and hormones on ^{3}H-uridine incorporation in pachytene spermatocytes and tubular fragments

5.2.1 Effect *in vitro* of the presence of Sertoli cells on ^{3}H-uridine incorporation in pachytene spermatocytes

Isolated spermatocytes are deprived from Sertoli cell influences, whereas spermatocytes in isolated tubular fragments may be influenced by the presence of the Sertoli cells. In order to detect a possible effect of the Sertoli cells on RNA synthesis in spermatocytes we have estimated the incorporation of ^{3}H-uridine in isolated pachytene spermatocytes which were preincubated either as isolated cells or present in tubular fragments. The incorporation of ^{3}H-uridine into RNA in the isolated pachytene spermatocytes was estimated after 0, 1 or 2 days of preincubation and the results are presented in Table I. In addition the percentage of isolated pachytene spermatocytes which did not incorporate ^{3}H-uridine was estimated using radioautography (Table I). Furthermore, electrophoretic profiles of labelled RNA from isolated pachytene spermatocytes were obtained (results not shown).

The incorporation of ^{3}H-uridine into RNA in isolated pachytene spermatocytes was decreased when the cells were preincubated either as isolated cells or in tubular fragments for 1-2 days. The incorporation of ^{3}H-uridine varied considerably when isolated spermatocytes were kept *in vitro* for 1 day. However, after preincubation under the different conditions the relative rate of ^{3}H-uridine incorporation (ratio of incorporated radioactivity to total uptake of radioactivity) was not markedly changed. Also, electrophoretic profiles of labelled RNA for spermatocytes preincubated for 1 day either as isolated cells or in tubular fragments and for freshly isolated cells were similar. The increased percentage of pa-

Table I Effect of preincubation on the incorporation of ^{3}H-uridine into RNA of isolated spermatocytes. Isolated spermatocytes were preincubated for 0, 1 or 2 days; tubular fragments were preincubated for 1 or 2 days. Spermatocytes were isolated from the tubular fragments after the preincubation. Subsequently, the isolated spermatocytes were incubated for 2 h in the presence of ^{3}H-uridine (10 µCi/ml, specific radioactivity 5 Ci/mmol) to estimate uptake and incorporation of ^{3}H-uridine (in triplo for each cell preparation). Incorporation is given as percentage of the incorporation at day 0 (100% = approximately 25,000 d.p.m./µg of DNA). Radioautograms were prepared to estimate the percentage of pachytene spermatocytes which actively incorporated ^{3}H-uridine.

preincubation conditions	preincubation period (days)	incorporation (%)	incorporation / uptake	inactive*** pachytene spermatocytes (%)
isolated spermatocytes*	0	100	0.36 ± 0.05 (5)	3 ± 2 (5)
	1	79 ± 43 (3)	0.39 ± 0.05 (3)	7 ± 3 (3)
	2**	43	0.31	18
tubular fragments	1	86 ± 5 (3)	0.45 ± 0.01 (3)	8; 10
	2	55	0.41	17

Values given are means ± S.D. (n) = number of different cell preparations.

*Results of the experiments in which radioautographical as well as biochemical estimations have been made are presented.

**Spermatocytes isolated from a total cell preparation by velocity sedimentation not before but after the preincubation period.

***Cells were considered to be inactive if the number of grains per nucleus was 10 or less.

chytene spermatocytes which were inactive after 1 or 2 days *in vitro* as well as changes in the uptake of ^{3}H-uridine can explain these results. Therefore, it can be suggested that the effect of preincubation on pachytene spermatocytes *in vitro* is comparable to that of hypophysectomy on the spermatocytes *in vivo*. In both cases the number of degenerating cells is increased whereas the synthesis of RNA in a part of the cell population is apparently not disturbed. However, the cellular uptake of the labelled precursor is possibly also influenced and this could be an early sign of degeneration.

5.2.2 Effect *in vitro* of hormones on ^{3}H-uridine incorporation in tubular fragments

No major alterations in RNA synthesis in pachytene spermatocytes seem to occur due to the absence of hormones *in vivo* after hypophysectomy (section 5.1). However, Parvinen & Söderström have observed an effect *in vitro* of hormones on RNA synthesis in tubular fragments at different stages of the cycle, isolated from adult rats 3 days after hypophysectomy. They reported that the rate of RNA synthesis was increased at all isolated stages within 2.5 h if the tubular fragments were incubated in the presence of follitropin plus testosterone (141). This result could reflect that the rate of RNA synthesis has increased in pachytene spermatocytes because these cells contribute significantly to the RNA synthesis of the tubular fragments, especially at stage VII (165). This observation could be in contrast to the present results on spermatocytes from hypophysectomized rats, which indicated that the relative rate of ^{3}H-uridine incorporation was not changed in the absence of hormones. Therefore the experiment as described by Parvinen & Söderström (141) was repeated using isolated tubular fragments (no selected stages) which were isolated from 30 or 60 day-old rats 2 days after hypophysectomy. The results presented in Table II show that the incorporation of ^{3}H-uridine varied considerably, but was not markedly influenced by the presence of the hormones. The ratio of the

Table II Effect of hormones on the incorporation of ^{3}H-uridine into RNA of isolated tubular fragments. Tubular fragments were isolated from 30 or 60 day-old rats, 3 days after hypophysectomy. The fragments were preincubated for 30 min without additions (control) or in the presence of follitropin (5 µg/ml), testosterone (0.1 µg/ml) or follitropin and testosterone, followed by incubation for 2 h under the same conditions in the presence of ^{3}H-uridine (10 µCi/ml, specific radioactivity 5 Ci/mmol). Uptake and incorporation of ^{3}H-uridine were estimated.

	Incorporated radioactivity (10^{-3} x d.p.m./µg of DNA)		$\frac{\text{Incorporated radioactivity}}{\text{Total uptake of radioactivity}}$	
age of rats (days)	30	60	30	60
control	7.5 ± 2.4	17.9 ± 6.2	0.27 ± 0.03	0.29 ± 0.03
follitropin	8.4 ± 4.1	14.8 ± 3.6	0.28 ± 0.03	0.27 ± 0.02
testosterone	7.1 ± 4.4	15.3 ± 3.8	0.29 ± 0.02	0.26 ± 0.04
follitropin and testosterone	10.4 ± 4.9	16.7 ± 4.4	0.29 ± 0.03	0.27 ± 0.04

Values given are means ± S.D. for n = 4-10 (number of incubations).

radioactivity incorporated to the total uptake of radioactivity was not changed when hormones were present in the incubation medium. These results reflect that the relative rate of ^{3}H-uridine incorporation in tubular fragments is not stimulated within 2½ hours by hormones *in vitro* under the conditions used in the present experiments.

In another experiment no effect *in vitro* of follitropin and testosterone could be shown on the incorporation of ^{3}H-uridine into 32S RNA in incubated tubular fragments (64). In conclusion, the present observations indicate that hormones do not induce changes in the incorporation of ^{3}H-uridine in incubated tubular fragments which could reflect changes in the activity of the pachytene spermatocytes. It is not known, however, whether the hormones could have a positive effect *in vitro* on the number of spermatocytes with apparent normal RNA synthesis in the incubated tubular fragments, similar to the positive effect *in vivo* of the hormones (section 5.1).

5.2.3 Effect of products secreted by Sertoli cells on ^{3}H-uridine incorporation in pachytene spermatocytes

The nature and function of the physical contacts between germinal cells and Sertoli cells is still a matter of debate. There is evidence that *in vivo* the germinal cells, spermatocytes included, are connected with Sertoli cells via junctional specialization of the membrane (153) and via gap junction-like structures (115). Also, some material may be transported between germinal cells and Sertoli cells via exocytosis and pinocytosis (47). However, these intercellular connections do not appear to occur very frequently (47,115). Hence, interaction between Sertoli cells and germinal cells could be achieved by intercellular transport of molecules which can pass plasma membranes (47). It seems also possible that proteins secreted by one cell type act on the other cell type via receptor-like interactions in the cell membrane (94).

In media from Sertoli cell cultures several different proteins, ABP included, are present which have been synthe-

Table III Effect of "Sertoli cell-conditioned" medium on the uptake and incorporation of ^{3}H-uridine by testicular cell types. Sertoli cells were incubated for 3 days without renewal of the medium, the medium was collected and the isolated testicular cell types were preincubated with this "conditioned" medium for 1 h, followed by incubation for 2 h with ^{3}H-uridine (10 µCi/ml) in the same medium. Controls were incubated in non-conditioned medium. Uptake and incorporation of uridine was estimated in triplo for each cell preparation and expressed as percentage of control values (100% = approximately 25,000 d.p.m./µg of DNA).

Uridine		Spermatocytes	Spermatids	Sertoli cells
2 µM	uptake	57 ± 18*	58	54
	incorporation	56 ± 19*	51	55
62 µM	uptake	102		
	incorporation	103		

*Mean ± S.D. for n = 7 (number of cells and "conditioned" medium preparations).

sized in the cells (149). The accumulation of these proteins in the medium can be stimulated by follitropin and testosterone (149). These proteins may be surface proteins which are shed from the cell membrane (30), but proteins can be actively secreted also via another mechanism. In addition, components other than proteins may be secreted by the cultured Sertoli cells. Such factors present in media from cultured Sertoli cells ("conditioned" medium) might influence the metabolism of isolated spermatocytes. We have therefore studied the possible effect of such "conditioned" media on the incorporation of ^{3}H-uridine into RNA of spermatocytes.

Sertoli cells were cultured in the presence of follitropin and testosterone and the medium was collected after 3 days. This "conditioned" medium was added in a 1:1 ratio to the incubation medium of isolated spermatocytes and after 1 h the uptake and incorporation of ^{3}H-uridine during 2 h was measured. The results are presented in Table III. The "conditioned" medium of Sertoli cells caused a decrease in uptake and incorporation of ^{3}H-uridine in spermatocytes. The same effect, however, was observed when the "conditioned" medium was added to cell preparations enriched in round spermatids or Sertoli cells. This inhibition of uptake and incorporation was not observed when the concentration of uridine in the incubation medium was increased from 2 µM up to 62 µM (see also Fig. 2 in section 4.1). Further it was observed that the inhibitor was stable at 100^{o}C and was dialyzable.

The inhibition does not seem to be cell-specific and may be caused by a competitive inhibitor of the uptake of uridine. Such a competitive inhibitor could be uridine or another nucleoside. In growing Hela cells, exposed to culture fluids from confluent Hela cell cultures, the incorporation of ^{3}H-uridine was also decreased (179) and it was concluded that this effect was probably due to the presence in the medium of uridine or a related compound, which resulted in a reduced uptake of ^{3}H-uridine, and was not the result of a reduced RNA synthesis (179). Our results on the inhibition of the incorporation of ^{3}H-uridine into RNA of spermatocytes might be

explained therefore via an inhibition of the uptake of ^{3}H-uridine, which does not reflect a specific effect of Sertoli cell medium components on the incorporation of ^{3}H-uridine into RNA of isolated spermatocytes.

5.3 Discussion

The uptake and incorporation of radioactively labelled uridine in pachytene spermatocytes was studied in an attempt to detect possible changes in RNA synthesis when the cells are exposed to different hormonal conditions or to influences of Sertoli cells. It was found for the spermatocytes that neither the relative rate of uridine incorporation nor the electrophoretic profiles of labelled RNA could be significantly changed. However, in the absence of hormones in vivo or when incubated in vitro an increased percentage of the isolated pachytene spermatocytes was degenerated and did not incorporate uridine. The uptake of uridine was probably also decreased and this decrease could mark the beginning of degeneration in a part of the cells.

Therefore there are no indications that the incorporation of ^{3}H-uridine into the main RNA fractions (heterogeneous RNA and rRNA) and the processing of RNA in the pachytene spermatocytes are under direct control of hormones and Sertoli cells. However, this does not necessarily apply to the synthesis and translation of mRNA. Progression of the meiotic prophase may be dependent on the synthesis of specific proteins and on the synthesis as well as the translation of specific mRNA molecules. Such mRNA molecules will constitute only a small fraction of the heterogeneous RNA. In order to study the mRNA molecules which are translated at the pachytene stage, it would be necessary to isolate the polysomal mRNA and to analyze electrophoretic profiles of proteins after translation of this mRNA in a translation system. Therefore, if hormones would influence the synthesis of mRNA, it could not have been detected with the techniques used in the present experiments.

Changes in biochemical processes in germinal cells after alterations in the (hormonal) environment may remain undetected for several other reasons as well. Dependent on the tissue used (testes from intact or hypophysectomized rats, incubated tubular fragments), a fluctuating number of degenerating spermatocytes sedimented with the intact spermatocytes during velocity sedimentation. This finding is in agreement with morphological observations on the degeneration of germinal cells *in vivo* at very low levels of follitropin and/or testosterone (36,155) which show that a part of the spermatocytes at the mid-pachytene stage is degenerating, whereas other pachytene spermatocytes at the same stage of development are intact. Therefore, it is difficult to isolate a homogeneous fraction of pachytene spermatocytes which contains cells that are all equally influenced by alterations in the environment. To study the earliest biochemical alterations in the pachytene spermatocytes, which certainly will preceed the visible degeneration of the cells after for example hypophysectomy, the procedure for cell isolation should be modified so that intact and degenerated cells are separated from the cells which are beginning to degenerate. The procedure which was used to isolate pachytene spermatocytes involves enzyme treatment, velocity sedimentation at unit gravity and several centrifugation steps. This cell isolation procedure may have removed or may have completely inactivated some of the degenerating cells. Therefore, the information obtained with such isolated spermatocytes does not necessarily reflect the degeneration of pachytene spermatocytes as it occurs *in vivo*.

This kind of problem could be prevented if it would be possible to influence RNA synthesis or other biochemical processes in germinal cells rapidly, because it is not likely that the composition of a germinal cell population is changed within a few hours. In relation to this, attempts have been made to estimate a possible rapid effect *in vitro* of hormones or media from cultured Sertoli cells on ^{3}H-uridine incorporation in isolated tubular fragments or pachytene spermatocytes

respectively (sections 5.2.2 and 5.2.3). No specific effects, however, were found.

In conclusion, the results presented in chapter 5 and appendix paper 3 suggest that RNA synthesis in a number of pachytene spermatocytes is not changed 2-4 days after hypophysectomy or 1-2 days after isolation of cells or tubular fragments. However, under these conditions a decrease of the total uptake and incorporation of ^{3}H-uridine in the spermatocyte preparations was found which can be explained by degenerative changes which take place in a part of the cell population. These results do neither suggest nor exclude the possibility that pachytene spermatocytes degenerate due to a defect in RNA synthesis. However, the primary defect in the pachytene spermatocytes which can lead to degeneration of these cells, as well as the role of hormones and Sertoli cells in such a process remain virtually unknown.

Chapter 6

GENERAL DISCUSSION

The work presented in this thesis was started to investigate a possible hormonal control of biochemical processes in germinal cells of the rat testis. In this connection, it was studied whether RNA synthesis in pachytene spermatocytes could be influenced by hormones or by factors from Sertoli cells. Such an influence, however, was not found. In cell preparations from hypophysectomized rats, the pachytene spermatocytes appeared to be present either as intact cells with apparently normal RNA synthesis or as degenerating cells with a more or less reduced uptake and incorporation of RNA precursors.

In this chapter the degeneration of germinal cells will be discussed because this appears to be a main feature in spermatogenesis (section 6.1). An essential part of spermatogenesis is the preparation for and the occurrence of meiotic divisions and a possible regulation of meiosis could be of great importance in the development of germinal cells (section 6.2). Finally, the interaction of hormones, Sertoli cells and germinal cells in the rat testis will be discussed in section 6.3.

6.1 Degeneration of male germinal cells

In testes of intact rats a small part of the germinal cell population degenerates at various steps of development, mainly during the spermatogonial stage and the first meiotic division (151). After hypophysectomy the degeneration of several types of germinal cells is drastically increased (19). At 5.5 days after hypophysectomy or 5 days after treatment with an antiserum for lutropin an increase was observed of the number of degenerating pachytene spermatocytes and of early and late spermatids at stage VII-VIII of the cycle of the seminiferous epithelium (36,155). These observations indicate that the most

marked effect of hypophysectomy on cell degeneration is not at random, but is probably restricted to a few steps in the development of the germinal cells.

Similarly, several examples listed below of deleterious effects of agents and treatments of widely different nature on the degeneration of germinal cells, show that different and selected steps in spermatogenesis can be influenced.

Spermatogenesis is very susceptible to heat and germinal cells are damaged when the temperature of the testis is slightly increased (the temperature of the testis in the scrotum of the rat is 32°C). For example, in a testis which is displaced from the scrotum to the abdomen (experimental cryptorchidism) mainly pachytene spermatocytes at stage I and II and early spermatids at stage I are damaged after 48 hours (140). Occlusion of the testicular artery caused specific disappearance of the cells undergoing mitosis or in the DNA-synthesizing period of meiosis (spermatogonia and preleptotene spermatocytes) (182). Chemical compounds can also affect defined steps in germinal cell development; for example dinitropyrroles and nitroimidazoles disturb primarily the development of the primary spermatocytes (142).

Some of the treatments which cause degeneration of male germinal cells have a direct effect on biochemical processes of the germinal cells. For example, protein synthesis in isolated immature spermatids was inhibited in the presence of 5-thio-D-glucose (135), a compound which induces sterility in mice and rats (72). Other treatments, however, may primarily affect the Sertoli cells which could subsequently result in degeneration of germinal cells. In this connection, it has been suggested that the Sertoli cells were damaged by an antispermatogenic agent (AF 1312/TS), as the primary cause of the degeneration of the germinal cells in the adluminal compartment of the tubules (28).

In spermatocytes, degeneration of the cells can be microscopically observed in the cytoplasm which is more darkly stained and which is more electron-dense in elec-

tronmicrographs, and in the nucleus which shows clumped chromatin. This reaction of the pachytene spermatocytes is observed after hypophysectony not in all cells but only in a part of the cell population present at the same site in the seminiferous epithelium and therefore it appears that the cells at the same stage of development do not degenerate simultaneously (155, appendix paper 3). This selective degeneration raises an intriguing question with respect to the mechanism which causes the degeneration.

Selective degeneration appears to be a common feature of mammalian male and female germinal cells. Mammalian oogonia and oocytes also degenerate at several steps in development; at the zygotene and the pachytene stage a part of the oocytes degenerates, whereas others remain intact (50). The oocytes might degenerate after errors in the formation of chiasmata (70). However, the primary cause and the mechanism of the selective degradation of oocytes as well as spermatocytes is still not known.

In summary, degeneration of germinal cells occurs at well defined steps in development. In addition, a selective degeneration is observed, particularly in pachytene spermatocytes, when hormone levels in vivo are severely reduced. A biochemical mechanism which could explain these observations is not known.

6.2 Possible regulation of meiosis

In fetal mammalian ovaries and testes the onset of meiosis is probably controlled by a meiosis-inducing substance as well as a meiosis-preventing substance (10) and the induction of meiosis can be achieved in these gonadal tissues in vitro (11,136). Moreover, in organ cultures of neonatal rat testes in chemically defined media pachytene spermatocytes are formed; however, such pachytene spermatocytes degenerate and are unable to complete the meiotic divisions (172,173). Even late pachytene spermatocytes do not complete meiotic divisions

when testicular tissue is cultured in media supplemented with hormones, sera or tissue extracts (175). In contrast, meiotic divisions can occur in vitro in insect spermatocytes and in microsporocytes from liliaceous plants but also in mammalian oocytes. Results obtained for these widely different systems could be used to explain the failure of mammalian spermatocytes to complete meiosis in vitro. Therefore these results will be discussed.

Several observations indicate that meiosis in meiocytes (microsporocytes) of liliaceous plants shows biochemical similarities to meiosis in mammalian spermatocytes (74,75,114). Meiocytes of liliaceous plants, explanted at the zygotene stage and cultured as coherent filaments of cells pass through the meiotic divisions in vitro in a chemically defined medium (79). However, the synchronous development which is found in intact anthers is disturbed. The tapetum, a layer of cells which envelops the meiocytes in the anthers, may provide a permissive environment for meiotic development but the tapetum probably does not play a role in inducing specific meiotic developments (79).

In organ cultures of fruitfly testes spermatocytes divide meiotically and elongating spermatids are formed in the presence of calf serum (61). Isolated germinal cysts of silkworm testes, which contain synchronized spermatocytes and somatic cyst cells, undergo meiosis and further spermatogenesis in vitro if the culture medium contains a "macromolecular factor" present in insect hemolymph (80). The factor is possibly related to serum factors present in calf serum and might act via the somatic cyst cells because isolated spermatocytes did not respond to the "macromolecular factor" and failed to develop (80).

Mammalian oocytes are arrested in the diplotene stage of the first meiotic division at prenatal or neonatal life. In the adult these diplotene (or dictyate) oocytes enter a growth phase and at a certain stage of oocyte growth they are able to undergo meiotic maturation, which is the progression from

diplotene up to metaphase II (169,183). Diplotene oocytes isolated at the beginning of the growth phase, fail to grow *in vitro* and degenerate (45). However, when oocytes are released from their follicles at the end of the growth phase, meiotic divisions in these isolated oocytes will occur in a chemically defined environment (65,161). During growth and maturation of the oocytes the qualitative pattern of protein components being synthesized changes (59,160,161). However, in fully-grown oocytes protein synthesis is not necessary for the dissolution of the nuclear membrane and condensation of the diplotene chromosomes into distinct bivalents which are the first events in meiotic maturation (188). These observations indicate that during the growth phase the oocytes are prepared for the meiotic divisions.

The pachytene stage in adult rat spermatocytes lasts for approx. 12 days, whereas the diplotene stage and the meiotic divisions proceed much more rapidly. Analogous to the induction of the meiotic divisions in insect germinal cysts by a "macromolecular factor", some factor might be required to complete the lengthy pachytene stage in mammalian spermatocytes and to start the processes leading to the meiotic divisions. Such a factor could be absent or inactive in cultures of mammalian testicular tissue.

On the other hand, the possibility should be considered that the pachytene spermatocytes cannot divide *in vitro*, because they are not well prepared for it. As was mentioned before, isolated fully-grown oocytes can start oocyte maturation even when protein synthesis is inhibited. However, for their growth the diplotene oocytes are dependent on the follicle cells. Similarly, pachytene spermatocytes could be highly dependent on the supporting action of fully-active Sertoli cells. In cultured mammalian testicular tissue, the Sertoli - germinal cell interaction is possibly disturbed and this may be the reason that the germinal cells do not complete meiosis but degenerate during the meiotic prophase.

In apparent contrast, the observations on meiocytes in liliaceous plants indicate that support from an extracellular source is not essential for the completion of meiosis in these cells. Therefore the meiotic divisions in these cells appear to occur on basis of a built-in program. In rats after hypophysectomy, when hormone levels are low and the activity of the Sertoli cell is probably reduced, a part of the pachytene spermatocytes can still produce spermatids (19). In view of this observation, it can be suggested that pachytene spermatocytes in rats can carry out the biochemical processes which are directly related to meiosis even when the Sertoli cells are not stimulated by hormones. Moreover, it appeared that after hypophysectomy RNA synthesis in a part of the pachytene spermatocytes was apparently normal (chapter 5, appendix paper 3).

In summary, it is still not clear whether a biochemical mechanism exists for the control of meiotic divisions in mammalian spermatocytes. It appears, however, that pachytene spermatocytes cannot further develop in testicular tissue in vitro and that a part of the germinal cell population degenerates after hypophysectomy. The primary cause for this is possibly not related to the biochemical processes which are directly related to meiosis.

6.3 Possible role of Sertoli cells and hormones in the development of male germinal cells

The close morphological association between Sertoli cells and germinal cells suggests a supporting function of Sertoli cells in spermatogenesis. For example, mitochondria in round spermatids are situated at the cell surface and smooth endoplasmic reticulum in the Sertoli cell cytoplasm has been observed in close association with the spermatid acrosome (48). The interaction may be achieved by intercellular transport of materials which can pass plasma membranes (section 5.2.3) and for this process the specific environment created by Sertoli

cells at the adluminal side of the blood-testis barrier (34, 88,93,185) could be important. In mouse germinal cells antigenic membrane components have been detected in the surface membranes of pachytene spermatocytes and more advanced germinal cells (126). These or similar proteins could cause the interaction between Sertoli cells and germinal cells.

Spermatogenic events do not occur at random but at well defined time intervals. Therefore some mechanism in the germinal epithelium should cause the coordination and sequential occurrence of, for example, the induction of meiosis at the spermatogonial stage, the meiotic divisions at the end of the meiotic prophase and spermatid differentiation. This regulation of spermatogenesis could be based on the interaction of Sertoli cells and germinal cells. However, the rate of development of the germinal cells is not influenced by hormones (17) and hormones may therefore not be involved in this type of regulation.

At low hormone levels the possible effect of the Sertoli cells on the germinal cells could be impaired and this might be the cause for degeneration of a part of the germinal cells. An increased degeneration of germinal cells at low hormone levels has been observed particularly for pachytene spermatocytes as well as round - and elongated spermatids at stage VII-VIII of the cycle of the seminiferous epithelium (36,155). The Sertoli cells during stage VII-VIII could be more dependent on hormones than the Sertoli cells at other stages. On the other hand, it can be suggested that the germinal cells at these stages make high demands on the supporting function of the Sertoli cell; at these stages the pachytene spermatocytes show high synthetic activities (130,166), the round spermatids are about to perform rotation and displacement of the nucleus, and the elongated spermatids are about to become released from the Sertoli cells (15).

In summary, the action of Sertoli cells on germinal cells could involve: 1. the maintenance of a specific environment in the seminiferous tubules; 2. the movement of the germinal

cells in the seminiferous epithelium; 3. the sequential occurrence of developmental steps in spermatogenesis. Up to now it is not known, however, whether Sertoli cells can influence biochemical processes in germinal cells which are related to the development of the germinal cells. In this connection it remains to be shown by which mechanism(s) Sertoli cells, in the presence of hormones, can act on the germinal cells so that degeneration of the germinal cells is prevented.

SUMMARY

Spermatogenesis in the rat is influenced by testosterone and follitropin (FSH). In the absence of these hormones germinal cells degenerate at various stages during development. It is not known, however, which biochemical processes are directly involved in the hormonal control of the development of male germinal cells. Therefore, the present work was started to study the possible effect of hormones on biochemical processes in germinal cells. Spermatogenesis and the hormonal control of spermatogenesis in the rat testis have been discussed in chapter 1. A description of the methods used for separation of germinal cells from rat testis and for analysis of RNA synthesis is given in chapter 2.

In experiments, described in chapter 3 and appendix paper 1, the following results were obtained:
1) A nuclear receptor for androgens, which could be demonstrated in Sertoli cells, was not present in spermatocytes and round spermatids. From this result, as well as from observations made by other authors, it has been concluded that Sertoli cells, but not the germinal cells, are target cells for androgens in the seminiferous epithelium.
2) The total activity per testis of β-glucuronidase and γ-glutamyltranspeptidase, enzymes which are mainly present in the Sertoli cells, increased during the development of the testis in immature rats. However, this change of total testicular enzyme activities was not clearly correlated with the appearance in the testis of specific germinal cell types during testis development.
3) In the present experiments, short-term treatment of rats with follitropin had no effect on the incorporation of ^{3}H-leucine (protein synthesis) in testicular tissue and isolated germinal cells.

The main part of the present work was performed with isolated cell preparations enriched in pachytene spermatocytes. The pachytene stage could represent a hormone-sensitive step

in spermatogenesis and RNA synthesis in the pachytene spermatocytes was studied because the action of hormones on many other cells is mediated via effects on RNA synthesis. From the results of experiments on the incorporation of radioactively labelled uridine into RNA of isolated pachytene spermatocytes, described in chapter 4 and appendix paper 2, it has been concluded that:

1) Isolated pachytene spermatocytes can be used for at least 8 h after isolation to study the incorporation of uridine into RNA of these cells.

2) The isolated pachytene spermatocytes incorporate ^{3}H-uridine mainly into RNA molecules with a heterogeneous electrophoretic mobility, designated as heterogeneous RNA. Most of this heterogeneous RNA in pachytene spermatocytes appeared to be labile. The synthesis of heterogeneous RNA in pachytene spermatocytes could play a role in the production of mRNA (precursor) molecules.

3) Isolated pachytene spermatocytes also synthesize tRNA and rRNA. Total rRNA appeared to be synthesized at a relatively low rate in the spermatocytes, when compared to the rate of rRNA synthesis in somatic cells. In addition, the formation of 28S rRNA from the 32S precursor in spermatocytes occurred much slower than the formation of 32S and 18S rRNA from the 45S precursor. There is no information about the possible significance of the retarded formation of 28S rRNA which apparently occurs in pachytene spermatocytes and possibly also in earlier germinal cell types, but not in round spermatids, Sertoli cells and peritubular cells.

Subsequent to the partial characterization of RNA synthesis in pachytene spermatocytes the possible effect of hormones or factors from Sertoli cells on the incorporation of radioactively labelled uridine into RNA of pachytene spermatocytes was studied (chapter 5 and appendix paper 3). These experiments have shown that:

1) The uptake and incorporation of ^{3}H-uridine in pachytene spermatocytes isolated from rat testes 2-4 days after hypophy-

sectomy was decreased as compared to the activity of spermatocytes isolated from intact rats. However, after hypophysectomy the rate of ^{3}H-uridine incorporation (ratio of incorporation and uptake) remained unchanged and also the qualitative electrophoretic profile of the synthesized heterogeneous RNA and rRNA in the pachytene spermatocytes was not affected. These results can be partly explained by the observations from radioautograms, that after hypophysectomy an increased percentage of the isolated pachytene spermatocytes did not incorporate ^{3}H-uridine. In the remaining cells the uptake of uridine could have been slightly influenced.

2) In pachytene spermatocytes 1-2 days after isolation, as well as in pachytene spermatocytes isolated from tubular fragments which were incubated for 1-2 days, the rate of ^{3}H-uridine incorporation was not markedly different from that in similar cells directly after isolation from the testis. However, changes in the uptake of ^{3}H-uridine were observed which could reflect cell degeneration and in autoradiograms an increased number of inactive cells was observed after 1-2 days of incubation. Components secreted by Sertoli cells had no specific effect on the rate of ^{3}H-uridine incorporation into RNA of pachytene spermatocytes.

From the results of the present experiments as well as from observations made by others on the role of hormones and Sertoli cells in spermatogenesis it has been concluded (see: chapter 6) that:

1) The presence of Sertoli cells is a prerequisite for the action of testosterone and follitropin on spermatogenesis because Sertoli cells are target cells for these hormones, whereas these hormones can probably not interact directly with germinal cells.

2) At very low hormone levels selective degeneration of germinal cells occurs. From the incorporation of radioactively labelled uridine into RNA of pachytene spermatocytes, it appeared that a part of the cell population is degenerating, whereas other pachytene spermatocytes are apparently not af-

fected. This selective degeneration forms a major problem when studying hormone effects in spermatogenesis.
3) It is not clear which mechanism(s) can prevent extensive degeneration of germinal cells via hormonal effects on Sertoli cells. Such a mechanism possibly does not involve the regulation of biochemical processes in the germinal cells which are directly related to the meiotic process or the regulation of RNA synthesis at the pachytene stage. After interaction with hormones, Sertoli cells can support the development of germinal cells. The function of Sertoli cells could involve the maintenance of a specific environment in the seminiferous tubules and the movement of the germinal cells within the seminiferous epithelium. Such activities might facilitate the development of germinal cells on basis of a built-in program in the germinal cells.

SAMENVATTING

De spermatogenese in de testis van de rat is afhankelijk van testosteron en follitropine (FSH). Wanneer deze hormonen niet in voldoende mate aanwezig zijn degenereren vele van de mannelijke geslachtscellen op verschillende momenten tijdens hun ontwikkeling. Het lijkt waarschijnlijk dat de hormonen biochemische processen in de mannelijke geslachtscellen beïnvloeden welke betrokken zijn bij de ontwikkeling van deze cellen. Het feit dat een dergelijke hormonale invloed nog niet was aangetoond vormde de aanleiding tot het werk dat in dit proefschrift is beschreven.

Een aantal gegevens over spermatogenese en de hormonale kontrole van de spermatogenese in de testis van de rat is besproken in hoofdstuk 1. De methoden die zijn gebruikt voor het isoleren van geslachtscellen uit de testis van de rat en voor het karakteriseren van RNA-synthese zijn beschreven in hoofdstuk 2.

De experimenten beschreven in hoofdstuk 3 en appendix-publikatie 1 hebben geleid tot de volgende resultaten:
1) Een receptor-eiwit specifiek voor androgene steroidhormonen kon worden aangetoond in kernen van Sertoli cellen, maar was afwezig in spermatocyten en ronde spermatiden. Deze resultaten maken het waarschijnlijk, dat niet de geslachtscellen maar wel de Sertoli cellen rechtstreeks door androgene steroiden worden beïnvloed.
2) De totale enzymaktiviteit per testis van β-glucuronidase en γ-glutamyltranspeptidase, enzymen die voornamelijk aanwezig zijn in de Sertoli cellen, nam toe tijdens de ontwikkeling van de testis in jonge ratten. Een duidelijk aanknopingspunt voor het onderzoek naar een funktioneel verband tussen veranderingen van de aktiviteit van deze enzymen en het verschijnen in de testis van pachyteen spermatocyten en spermatiden was echter niet aan te geven.
3) Na inspuiting van follitropine in jonge ratten was er geen snelle verandering meetbaar van de inbouw van ^{3}H-leucine in eiwitten in testisweefsel en geïsoleerde geslachtscellen.

Het pachyteenstadium van de meiose in spermatocyten is waarschijnlijk een van de meest hormoon-gevoelige stadia van de spermatogenese. Om deze reden werd voor de meeste experimenten die in dit proefschrift zijn beschreven gebruik gemaakt van geïsoleerde celpreparaten, verrijkt met pachyteen spermatocyten. Voor biochemisch onderzoek naar de effekten van hormonen op deze cellen viel de keuze op het karakteriseren van de synthese van RNA. Experimenten over de inbouw van radioaktief gemerkt uridine in RNA van geïsoleerde pachyteen spermatocyten (hoofdstuk 4, appendixpublikatie 2) hebben de volgende resultaten opgeleverd:

1) Geïsoleerde pachyteen spermatocyten kunnen gedurende tenminste 8 uur na isolatie worden gebruikt om de inbouw van ^{3}H-uridine in RNA te meten.

2) In geïsoleerde spermatocyten werd ^{3}H-uridine vooral ingebouwd in heterogeen RNA, maar het grootste gedeelte van dit heterogene RNA werd weer snel afgebroken. De synthese van heterogeen RNA in pachyteen spermatocyten kan van belang zijn voor de produktie van mRNA.

3) In geïsoleerde pachyteen spermatocyten werd ^{3}H-uridine tevens ingebouwd in rRNA en tRNA. De relatieve snelheid van rRNA-synthese was laag in vergelijking met het verschijnen van radioaktief gemerkt rRNA in somatische cellen. Het 45S rRNA werd normaal gesplitst in 32S en 18S rRNA maar de synthese van 28S rRNA was sterk vertraagd. Deze langzame synthese van 28S rRNA treedt mogelijk ook op in de geslachtscellen in stadia voorafgaand aan het pachyteenstadium maar niet in ronde spermatiden, Sertoli cellen en peritubulaire cellen. Een funktie voor de vertraagde vorming van 28S rRNA is niet bekend.

Vervolgens werd de mogelijke invloed van hormonen en faktoren afkomstig van Sertoli cellen op de inbouw van radioaktief gemerkt uridine in RNA van pachyteen spermatocyten onderzocht (hoofdstuk 5 en appendixpublikatie 3). Uit de resultaten van deze experimenten werd het volgende gekonkludeerd:

1) De opname en inbouw van ^{3}H-uridine in preparaten van geïsoleerde pachyteen spermatocyten waren sterk verminderd wanneer de cellen 2-4 dagen na hypofysektomie van de ratten werden geïsoleerd. De hypofysektomie had echter geen effekt op de relatieve inbouwsnelheid van het ^{3}H-uridine (ratio van de inbouw en opname) en op het elektroforeseprofiel van radioaktief gemerkt heterogeen RNA en rRNA. Deze resultaten konden ten dele worden verklaard door waarnemingen gedaan met autoradiografie, waarbij bleek dat na hypofysektomie een verhoogd percentage van de geïsoleerde pachyteen spermatocyten geen ^{3}H-uridine had ingebouwd, terwijl door de overige cellen wel, maar mogelijk iets minder, ^{3}H-uridine werd opgenomen en ingebouwd.
2) De relatieve snelheid waarmee ^{3}H-uridine werd ingebouwd in RNA van geïsoleerde spermatocyten was niet opvallend veranderd wanneer deze inbouw werd gemeten 1-2 dagen na isolatie van de cellen of nadat de cellen gedurende 1-2 dagen waren geïnkubeerd in geïsoleerde stukjes tubuli, vergeleken met de inbouwsnelheid in direkt uit de testis geïsoleerde spermatocyten. Veranderingen van de totale opname en inbouw van ^{3}H-uridine en een toename van het aantal inaktieve spermatocyten na 1-2 dagen inkubatie wijzen echter op degeneratie van een gedeelte van de spermatocytenpopulatie. Een specifiek effekt van komponenten in het medium van gekweekte Sertoli cellen op de inbouw van ^{3}H-uridine in RNA van geïsoleerde pachyteen spermatocyten werd niet gevonden.

De resultaten van de experimenten beschreven in dit proefschrift en waarnemingen beschreven in de literatuur (hoofdstuk 6) hebben geleid tot de volgende konklusies over de rol van hormonen en Sertoli cellen in de spermatogenese.
1) Testosteron en follitropine kunnen de spermatogenese alleen beïnvloeden wanneer Sertoli cellen een interaktie kunnen aangaan met de geslachtscellen, omdat deze hormonen een rechtstreeks effekt hebben op Sertoli cellen, maar waarschijnlijk niet op de geslachtscellen.
2) Wanneer niet voldoende hormoon aanwezig is, degenereren de

geslachtscellen op een selektieve wijze. Gebleken is, onder andere door het meten van de inkorporatie van radioaktief gemerkt uridine in RNA van pachyteen spermatocyten, dat een gedeelte van de cellen degenereert, terwijl andere pachyteen spermatocyten blijkbaar niet zijn beïnvloed. Deze selektieve degeneratie vormt een belangrijk probleem bij het bestuderen van het effekt van hormonen op de spermatogenese.

3) Het is niet bekend op welke wijze hormonen via Sertoli cellen de ontwikkeling van de geslachtscellen beïnvloeden. Mogelijk hebben Sertoli cellen geen regulerende invloed op biochemische processen die direkt betrokken zijn bij de meiose of op de RNA-synthese in het pachyteenstadium maar wel een funktie bij het handhaven van een specifiek milieu in de seminifere tubuli en voor het verplaatsen van de geslachtscellen in het seminifere epitheel. Het is niet onwaarschijnlijk dat door deze aktiviteit van Sertoli cellen de geslachtscellen zich op basis van een ingebouwd programma kunnen ontwikkelen.

REFERENCES

1. Ahmad, N., G.C. Haltmeyer and K.B. Eik-Nes (1975) J. Reprod. Fert. 44, 103.
2. Anderson, D.M. and L.D. Smith (1977) Cell 11, 663.
3. Bellvé, A.R., J.C. Cavicchia, C.F. Millette, P.A. O'Brien, Y.M. Bhatnagar and M. Dym (1977) J. Cell Biol. 74, 68.
4. Berg, J.A. van den, M. Gruber and G. AB (1976) FEBS Lett. 63, 65.
5. Billing, R.J., B. Barbiroli and R.M.S. Smellie (1969) Biochim. biophys. Acta 190, 52.
6. Blackshaw, A.W. and J.S.H. Elkington (1970) J. Reprod. Fert. 22, 69.
7. Borek, E. and P.R. Srinivasan (1966) Annual Rev. Biochem. 35, 275.
8. Bucci, S., I. Nardi, G. Mancino and L. Fiume (1971) Expl. Cell Res. 69, 462.
9. Bullock, L.P. and C.W. Bardin (1974) Endocrinology 94, 746.
10. Byskov, A.G. (1978) Ann. Biol. anim. Biochim. Biophys., in press.
11. Byskov, A.G. and L. Saxen (1976) Devl. Biol. 52, 193.
12. Castro, A.E., A. Alonso and R.E. Mancini (1972) J. Endocr. 52, 129.
13. Chandley, A.C., Y. Hotta and H. Stern (1977) Chromosoma 62, 243.
14. Christensen, A.K. and N.R. Mason (1965) Endocrinology 76, 646.
15. Clermont, Y. (1972) Physiol. Rev. 52, 198.
16. Clermont, Y. and E. Bustos-Obregon (1968) Am. J. Anat. 122, 237.
17. Clermont, Y. and S.C. Harvey (1965) Endocrinology 76, 80.
18. Clermont, Y. and S.C. Harvey (1967) Ciba Fdn. Colloq. Endocr. 16, 173.
19. Clermont, Y. and H. Morgentaler (1955) Endocrinology 57, 369.

20. Clermont, Y. and B. Perey (1957) Am. J. Andr. 100, 241.
21. Courot, M., M.-T. Hochereau de Reviers and R. Ortavant (1970) in The Testis, Vol. I (A.D. Johnson, W.R. Gomes and N.L. Vandemark, eds.) p. 339, Academic Press, New York and London.
22. Crippa, M. (1970) Nature 227, 1138.
23. Dabeva, M.D., K.P. Dudov, A.A. Hadjiolov, I. Emanuilov and B.N. Todorov (1976) Biochem. J. 160, 495.
24. Davidson, E.H. and B.R. Hough (1971) J. Molec. Biol. 56, 491.
25. Davies, A.G., W.E. Davies and C. Summer (1975) J. Reprod. Fert. 42. 415.
26. Davies, A.G. and N.R. Lawrence (1976) J. Physiol., Lond. 257, 18p.
27. Davis, J.C. and A.W. Schuetz (1975) Expl. Cell Res. 91, 79.
28. De Martino, C., M. Stefanini, A. Agrestini, D. Cocchia, M. Morelli and P. Scorza Barcellona (1975) Expl. molec. Path. 23, 321.
29. Dessen, E.M.B. and A.L.P. Perondini (1976) Cell Differentiation 5, 275.
30. Doljanski, F. and M. Kapeller (1976) J. theor. Biol. 62, 253.
31. Dorrington, J.H. and D.T. Armstrong (1975) Proc. natn. Acad. Sci. USA 72, 2677.
32. Dorrington, J.H., N.F. Roller and I.B. Fritz (1975) Molec. cell. Endocr. 3, 57.
33. Dulbecco, R. and M. Vogt (1954) J. exp. Med. 99, 167.
34. Dym, M. and D.W. Fawcett (1970) Biol. Reprod. 3, 308.
35. Dym, M. and D.W. Fawcett (1971) Biol. Reprod. 4, 195.
36. Dym, M. and H.G. Madhwa Raj (1977) Biol. Reprod. 17, 676.
37. Eagle, H. (1959) Science 130, 432.
38. Earle, W.R. (1943) J. natn. Cancer Inst. 4, 165.
39. Eik-Nes, K.B. (1970) in The Androgens of the Testis (K.B. Eik-Nes, ed.) p. 1, Marcel Dekker, New York.
40. Elkington, J.S.H. (1975) Molec. cell. Endocr. 2, 157.

41. Elkington, J.S.H. and A.W. Blackshaw (1970) J. Reprod. Fert. 23, 1.
42. Elkington, J.S.H., A.W. Blackshaw and B. de Jong (1973) Aust. J. biol. Sci. 26, 491.
43. Elkington, J.S.H. and A.W. Blackshaw (1974) Aust. J. biol. Sci. 27, 59.
44. Elkington, J.S.H. and A.W. Blackshaw (1974) Aust. J. biol. Sci. 27, 267.
45. Eppig, J.J. (1977) Devl. Biol. 60, 371.
46. Fakunding, J.L., D.J. Tindall, J.R. Dedman, C.R. Mena and A.R. Means (1976) Endocrinology 98, 392.
47. Fawcett, D.W. (1974) in Male Fertility and Sterility (R.E. Mancini and L. Martini, eds.) p. 13, Academic Press, London, New York and San Francisco.
48. Fawcett, D.W. (1975) in Handbook of Physiology, Section 7, Vol. 5 (D.W. Hamilton and R.O. Greep, eds.) p. 21, American Physiological Society, Washington D.C.
49. Fawcett, D.W., W.B. Neaves and M.N. Flores (1973) Biol. Reprod. 9, 500.
50. Franchi, L.L. and T.G. Baker (1973) in Human Reproduction (E.S.E. Hafez and T.N. Evans, eds.) p. 53, Harper and Row Publishers Inc., London.
51. Fritz, I.B., F.F.G. Rommerts, B.G. Louis and J.H. Dorrington (1976) J. Reprod. Fert. 46, 17.
52. Fuhrman, S.A. and G.N. Gill (1974) Endocrinology 94, 691.
53. Galdieri, M. and V. Monesi (1974) Expl. Cell Res. 85, 287.
54. Galena, H.J., A.K. Pillai and C. Terner (1974) J. Endocr. 63, 223.
55. Geremia, R. (1978) Expl. Cell Res. 111, 23.
56. Geremia, R., C. Boitani, M. Conti and V. Monesi (1977) Cell Differentiation 5, 343.
57. Geremia, R., R.B. Goldberg and W.R. Bruce (1976) Andrologia 8, 147.
58. Go, V.L.W., R.G. Vernon and I.B. Fritz (1971) Can. J. Biochem. 49, 761.
59. Golbus, M.S. and M.P. Stein (1976) J. exp. Zool. 198, 337.

60. Goldberg,R.B., R. Geremia and W.R. Bruce (1977) Differentiation 7, 167.
61. Gould-Somero, M. and L. Holland (1974) Wilhelm Roux Arch. 174, 133.
62. Grimes, S.R. Jr., M.L. Meistrich, R.D. Platz and L.S. Hnilica (1977) Expl. Cell Res. 110, 31.
63. Griswold, M.D., E.R. Mably and I.B. Fritz (1976) Molec. cell. Endocr. 4, 139.
64. Grootegoed, J.A., F.F.G. Rommerts and H.J. van der Molen (1978) in Hormones and Cell Regulation, Vol. 2 (J. Dumont and J. Nunez, eds.) p. 55, North-Holland Publishing Company, Amsterdam, New York and Oxford.
65. Gwatkin., R.B.L. and O.F. Andersen (1976) Life Sci. 19, 527.
66. Hägenas, L., E.M. Ritzén, L. Ploën, V. Hansson, F.S. French and S.N. Nayfeh (1975) Molec. cell. Endocr. 2, 339.
67. Hallberg, R.L. and D.C. Smith (1976) Devl. Biol. 48, 308.
68. Hanks, J.H. and R.E. Wallace (1949) Proc. Soc. exp. Biol. Med. 71, 196.
69. Hansson, V., W.S. McLean, A.A. Smith, D.J. Tindall, S.C. Weddington, S.N. Nayfeh and F.S. French (1974) Steroids 23, 823.
70. Henderson, S.A. and R. Edwards (1968) Nature 218, 22.
71. Hodgen, G.D. and R.J. Sherins (1973) Endocrinology 93, 985.
72. Homm, R.E. C. Rusticus and D.W. Hahn (1977) Biol. Reprod. 17, 697.
73. Honma, Y. and T. Noumura (1975) J. Endocr. 65, 377.
74. Hotta, Y., A.C. Chandley and H. Stern (1977) Nature 269, 240.
75. Hotta, Y., A.C. Chandley and H. Stern (1977) Chromosoma 62, 255.
76. Huckins, C., N. Mills, P. Besch and A.R. Means (1973) Endocrinology 92, A-94.
77. Iatrou, K. and G.H. Dixon (1977) Cell 10, 433.

78. Irusta, O. and G.F. Wassermann (1974) J. Endocr. 60, 463.
79. Ito, M. and H. Stern (1967) Devl. Biol. 16, 36.
80. Kambysellis, M.P. and C.M. Williams (1971) Biol. Bull. 141, 527.
81. Keyl, H.-G. (1975) Chromosoma 51, 75.
82. Kierszenbaum, A.L. (1974) Biol. Reprod. 11, 365.
83. Kierszenbaum, A.L. and L.L. Tres (1974) J. Cell Biol. 60, 39.
84. Kierszenbaum, A.L. and L.L. Tres (1974) J. Cell Biol. 63, 923.
85. Kierszenbaum, A.L. and L.L. Tres (1975) J. Cell Biol. 65, 258.
86. King, R.J.B. and W.I.P. Mainwaring (1974) Steroid-Cell Interactions, Butterworths, London.
87. Knowler, J.T. (1976) Eur. J. Biochem. 64, 161.
88. Koskimies, A.I. and M. Kormano (1973) J. Reprod. Fert. 34, 433.
89. Lam, D.M.K., R. Furrer and W.R. Bruce (1970) Proc. natn. Acad. Sci. USA 65, 192.
90. Lawrence, N.R. and A.G. Davies (1977) J. Reprod. Fert. 49, 41.
91. Leblond, C.P. and Y. Clermont (1952) Ann. N.Y. Acad. Sci. 55, 548.
92. León, P.E. (1975) J. theor. Biol. 55, 481.
93. Levine, N. and D.J. Marsh (1971) J. Physiol. 213, 557.
94. Levine, S., R. Pictet and W.J. Rutter (1973) Nature New Biol. 246, 49.
95. Lewicki, P.P. and A.J. Sinskey (1970) Analyt. Biochem. 33, 273.
96. Lewin, B. (1975) Cell 4, 11.
97. Loening, U.E. (1969) Biochem. J. 113, 131.
98. Loir, M. (1972) Ann. Biol. anim. Biochim. Biophys. 12, 203.
99. Loir, M. and M. Lanneau (1974) Expl. Cell Res. 83, 319.
100. Loir, M. and M. Lanneau (1975) Experientia 31, 401.
101. Lostroh, A.J. (1963) Acta endocr. 43, 592.

102. Lostroh, A.J. (1969) Endocrinology 85, 438.
103. Louis, B.G. and I.B. Fritz (1977) Molec. cell. Endocr. 7, 9.
104. Lowry, D.N., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) J. biol. Chem. 193, 265.
105. Lu, C. and A. Steinberger (1977) Biol. Reprod. 17, 84.
106. Lyon, M.F. (1972) Biol. Rev. 47, 1.
107. Lyon, M.F., P.H. Glenister and M.L. Lamoreux (1975) Nature 258, 620.
108. Lyon, M.F. and S.G. Hawkes (1970) Nature 227, 1217.
109. Madhwa Raj, H.G. and M. Dym (1976) Biol. Reprod. 14, 489.
110. Mainwaring, W.I.P. (1977) The Mechanism of Action of Androgens, Springer-Verlag, New York, Heidelberg and Berlin.
111. Mainwaring, W.I.P., F.R. Mangan, R.A. Irving and D.A. Jones (1974) Biochem. J. 144, 413.
112. Majumder, G.C., S. Lessin and R.W. Turkington (1975) Endocrinology 96, 890.
113. Males, J.L. and R.W. Turkington (1971) Endocrinology 88, 579.
114. Mather, J. and Y. Hotta (1977) Expl. Cell Res. 109, 181.
115. McGinley, D., Z. Posalaky and M. Porvaznik (1977) Anat. Rec. 189, 211.
116. Means, A.R. (1971) Endocrinology 89, 981.
117. Means, A.R. and P.F. Hall (1967) Endocrinology 81, 1151.
118. Means, A.R. and P.F. Hall (1968) Endocrinology 82, 597.
119. Means, A.R. and C. Huckins (1974) in Hormone Binding and Target Cell Activation in the Testis (M. Dufau and A. Means, eds.) p. 145, Plenum Press, New York.
120. Means, A.R., E. MacDougall, T.R. Soderling and J.D. Corbin (1974) J. biol. Chem. 249, 1231.
121. Means, A.R. and J. Vaitukaitis (1972) Endocrinology 90, 39.
122. Meistrich, M.L., W.R. Bruce and Y. Clermont (1973) Expl. Cell Res. 79, 213.

123. Meistrich, M.L., B.O. Reid and W.J. Barcellona (1975) J. Cell Biol. 64, 211.
124. Meistrich, M.L., P.K. Trostle, M. Frapart and R.P. Erickson (1977) Devl. Biol. 60, 428.
125. Miller, R.G. and R.A. Phillips (1969) J. cell. Physiol. 73, 191.
126. Millette, C.F. and A.R. Bellvé (1977) J. Cell Biol. 74, 86.
127. Mills, N.C. and A.R. Means (1972) Endocrinology 91, 147.
128. Mills, N.C., T.M. Mills and A.R. Means (1977) Biol. Reprod. 17, 124.
129. Monesi, V. (1965) Chromosoma 17, 11.
130. Monesi, V. (1965) Expl. Cell Res. 39, 197.
131. Moore, G.P.M. (1971) Expl. Cell Res. 68, 462.
132. Mulder, E., M.J. Peters, J. de Vries and H.J. van der Molen (1975) Molec. cell. Endocr. 2, 171.
133. Mulder, E., M.J. Peters, W.M.O. van Beurden, M. Galdieri, F.F.G. Rommerts, F.H.A. Janszen and H.J. van der Molen (1976) J. Endocr. 70, 331.
134. Muramatsu, M., T. Utakoji and H. Sugano (1968) Expl. Cell Res. 53, 278.
135. Nakamura, M. and P.F. Hall (1977) J. Reprod. Fert. 49, 395.
136. O, W.-S. and T.G. Baker (1976) J. Reprod. Fert. 48, 399.
137. Ohno, S. (1971) Nature 234, 134.
138. Orth, J. and A.K. Christensen (1977) Endocrinology 101, 262.
139. Parish, J.H. and K.S. Kirby (1966) Biochim. biophys. Acta 129, 554.
140. Parvinen, M. (1973) Virchows Arch. Abt. B Zellpath. 13, 38.
141. Parvinen, M. and K.-O. Söderström (1976) J. Steroid Biochem. 7, 1021.
142. Patanelli, D.J. (1975) in Handbook of Physiology, Section 7 (D.W. Hamilton and R.O. Greep, eds.), p. 245, American Physiological Society, Washington D.C.

143. Perry, R.P. and D.E. Kelley (1970) J. cell. Physiol. 76, 127.
144. Peterson, E.A. and W.H. Evans (1967) Nature 214, 824.
145. Platz, R.D., S.R. Grimes, M.L. Meistrich and L.S. Hnilica (1975) J. biol. Chem. 250, 5791.
146. Reddy, P.R.K. and C.A. Villee (1975) Biochem. biophys. Res. Commun. 63, 1063.
147. Rillema, J.A. (1975) Endocrinology 96, 1307.
148. Rogers, R.E. and L.W. Browder (1977) Devl. Biol. 55, 148.
149. Rommerts, F.F.G., B.Ch. Krüger-Sewnarain, A. van Woerkom-Blik, J.A. Grootegoed and H.J. van der Molen (1978) Molec. cell. Endocr. 10, 39.
150. Romrell, L.J., A.R. Bellvé and D.W. Fawcett (1976) Devl. Biol. 49, 119.
151. Roosen-Runge, E.C. (1973) J. Reprod. Fert. 35, 339.
152. Rooij, D.G. de (1969) Z. Zellforsch. 99, 134.
153. Ross, M.H. and J. Dobler (1975) Anat. Rec. 183, 267.
154. Russell, L. (1977) Am. J. Anat. 148, 313.
155. Russell, L. and Y. Clermont (1977) Anat. Rec. 187, 347.
156. Sanborn, B.M., A. Steinberger, M.L. Meistrich and E. Steinberger (1975) J. Steroid Biochem. 6, 1459.
157. Saxena, B.B. and P. Rathnam (1976) in Advances in Sex Hormone Research, Vol. 2 (R.L. Singhal and J.A. Thomas, eds.) p. 289, HM + M Medical and Scientific Publishers, England.
158. Scherrer, K., G. Spohr, N. Granboulan, C. Morel, J. Grosclaude and C. Chezzi (1970) Cold Spring Harb. Symp. quant. Biol. 35, 539.
159. Schuetz, A.W. (1974) Biol. Reprod. 10, 150.
160. Schultz, R.M. and P.M. Wassarman (1977) Proc. natn. Acad. Sci. USA 74, 538.
161. Schultz, R.M. and P.M. Wassarman (1977) J. Cell Sci. 24, 167.
162. Setchell, B.P., B.T. Hinton, F. Jacks and R.V. Davies (1976) Molec. cell. Endocr. 6, 59.
163. Shen, R.-S. and I.P. Lee (1976) J. Reprod. Fert. 48, 301.

164. Shiokawa, K., A. Kawahara, Y. Misumi, Y. Yasuda and K. Yamana (1977) Devl. Biol. 57, 210.
165. Söderström, K.-O. (1976) Expl. Cell Res. 102, 237.
166. Söderström, K.-O. and M. Parvinen (1976) Molec. cell. Endocr. 5, 181.
167. Söderström, K.-O. and M. Parvinen (1976) Hereditas 82, 25.
168. Sommerville, J. and D.B. Malcolm (1976) Chromosoma 55, 183.
169. Sorensen, R.A. and P.M. Wassarman (1976) Devl. Biol. 50, 531.
170. Stanley, W.M. and R.M. Bock (1965) Biochemistry 4, 1302.
171. Steinberger, A., J.J. Heindel, J.N. Lindsey, J.S.H. Elkington, B.M. Sanborn and E. Steinberger (1975) Endocr. Res. Commun. 2, 261.
172. Steinberger, A. and E. Steinberger (1970) in The Testis, Vol. II (A.D. Johnson, W.R. Gomes and N.L. VandeMark, eds.), p. 363, Academic Press, New York and London.
173. Steinberger, A. and E. Steinberger (1970) in Advances in Tissue Culture, Vol. 5 (C. Waymouth, ed.), p. 17, The Williams and Wilkins Company, Baltimore.
174. Steinberger, E. (1971) Physiol. Rev. 51, 1,
175. Steinberger, E., A. Steinberger and M. Ficher (1970) in Recent Progress in Hormone Research, Vol. 26 (E.B. Astwood, ed.) p. 547, Academic Press, New York and London.
176. Swann, P.F., A.C. Peacock and S. Bunting (1975) Biochem. J. 150, 335.
177. Szasz, G. (1969) Clin. Chem. 15, 124.
178. Tavitian, A., S.C. Kretsky and G. Acs (1969) Biochim. Biophys. Acta 179, 50.
179. Tilley, R. and C.N. Nair (1975) J. Cell Physiol. 86, 359.
180. Tindall, D.J., G.R. Cunningham and A.R. Means (1978) J. biol. Chem. 253, 166.
181. Tindall, D.J. and A.R. Means (1976) Endocrinology 99, 809.

182. Tjioe, D.Y. and E. Steinberger (1970) J. Reprod. Fert. 21, 489.
183. Tsafriri, A., M.A. Lieberman, A. Barnea, S. Bauminger and H.R. Lindner (1973) Endocrinology 93, 1378.
184. Tsai, Y.-H., B.M. Sanborn, A. Steinberger and E. Steinberger (1977) Biochem. biophys. Res. Commun. 75, 366.
185. Tuck, R.R., B.P. Setchell, G.M.H. Waites and J.A. Young (1970) Pflügers Arch. 318, 225.
186. Tung, P.S., J.H. Dorrington and I.B. Fritz (1975) Proc. natn. Acad. Sci. USA 72, 1838.
187. Utakoji, T. (1966) Expl. Cell Res. 42, 585.
188. Wassarman, P.M., W.J. Josefowicz and G.E. Letourneau (1976) J. Cell Sci. 22, 531.
189. Weddington, S.C., V. Hansson, E.M. Ritzén, L. Hägenas, F.S. French and S.N. Nayfeh (1975) Nature 254, 145.
190. Weinberg, R.A. (1973) Annual Rev. Biochem. 42, 329.
191. Welsh, M.J. and J.P. Wiebe (1975) Endocrinology 96, 618.
192. Wilkinson, D.S. and H.C. Pitot (1973) J. biol. Chem. 248, 63.
193. Williamson, B. (1976) Nature 264, 397.
194. Winer, A.D. and M.B. Nikitovitch-Winer (1971) FEBS Lett. 16, 21.
195. Woo, S.L.C. and B.W. O'Malley (1975) Life Sci. 17, 1039.
196. Xuma, M. and R.W. Turkington (1972) Endocrinology 91, 415.

LIST OF TRIVIAL NAMES AND ENZYMES

Trivial names frequently used in this work	Systematic names
deoxyribonuclease	- deoxyribonucleate oligonucleotide-hydrolase (EC 3.1.4.5)
dihydrotestosterone	- 17β-hydroxy-5α-androstan-3-one
β-glucuronidase	- β-D-glucuronide glucuronohydrolase (EC 3.2.1.31)
γ-glutamyltranspeptidase	- (γ-glutamyl)-peptide: amino-acid γ-glutamyltransferase (EC 2.3.2.2)
testosterone	- 17β-hydroxy-4-androsten-3-one
trypsin	- trypsin (EC 3.4.4.4)

LIST OF ABBREVIATIONS

A_{260}	- absorbance at 260 nm
ABP	- androgen binding protein
BSA	- bovine serum albumin
Ci	- curie
cyclic AMP	- adenosine 3':5'-monophosphoric acid, cyclic
DNA	- deoxyribonucleic acid
DNAase	- deoxyribonuclease
d.p.m.	- disintegrations per minute
E.coli	- Escherichia coli
FSH	- follitropin (follicle stimulating hormone)
g	- relative centrifugal force
h	- hour
hnRNA	- heterogeneous nuclear RNA
i.u.	- international unit
l	- litre
LH	- lutropin (luteinizing hormone)
M	- moles per litre
min	- minute
mol.wt.	- molecular weight
mRNA	- messenger RNA
n	- number of estimations
rDNA	- ribosomal DNA
RNA	- ribonucleic acid
r.p.m.	- revolutions per minute
rRNA	- ribosomal RNA
S	- Svedberg unit
S.D.	- standard deviation
Tris	- 2-amino-2-hydroxymethylpropane-1,3-diol
tRNA	- transfer RNA

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Anton Grootegoed

CURRICULUM VITAE

Op 11 mei 1950 werd J.A. Grootegoed te Amsterdam geboren. In 1967 behaalde hij het getuigschrift HBS-B aan het Heldring College te Zetten. De studie biologie aan de Katholieke Universiteit te Nijmegen werd begonnen in 1967 en voltooid in 1973 met het cum laude behalen van het doctoraal examen met als hoofdvak genetica. In september 1973 volgde een aanstelling als wetenschappelijk medewerker bij de afdeling Biochemie II van de Faculteit der Geneeskunde van de Erasmus Universiteit te Rotterdam, waar het in dit proefschrift beschreven onderzoek werd verricht.

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ABSENCE OF A NUCLEAR ANDROGEN RECEPTOR IN ISOLATED GERMINAL CELLS OF RAT TESTIS

J.A. GROOTEGOED, M.J. PETERS, E. MULDER, F.F.G. ROMMERTS and H.J. VAN DER MOLEN

Department of Biochemistry (Division of Chemical Endocrinology), Medical Faculty, Erasmus University Rotterdam, Rotterdam, The Netherlands

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Androgen receptors are known to be present in the seminiferous tubules of rat testis and in the present study it has been attempted to compare the binding of [^{3}H]testosterone to androgen receptors in male germinal cells and Sertoli cells. Cell preparations enriched in germinal cells and Sertoli cells were isolated from testicular tissue of 30–35-day-old rats. The cell preparations were either obtained from intact rats and labelled in vitro with [^{3}H]testosterone or were obtained from the testes of hypophysectomized rats which were labelled in vivo with [^{3}H]testosterone prior to the isolation of the cells. The nuclear fractions of the labelled cell preparations were extracted with 0.4 M KCl and the extracts were fractionated by sucrose density gradient centrifugation to estimate specific binding of radioactive steroid.

Specific binding of radioactive steroid to nuclear androgen receptors was observed in Sertoli cell preparations but not in preparations of germinal cells (spermatocytes and round spermatids).

Keywords: androgen receptor; testis; germinal cells; Sertoli cells.

In the male rat spermatogenesis is dependent on the presence of testosterone (Clermont and Harvey, 1967). The localization of androgen receptors could help to define the cell types of the seminiferous epithelium which might be influenced directly by testosterone. It has been previously shown that the tubular compartment of the testis of mature rats contains a nuclear androgen receptor which binds mainly testosterone (Mulder et al., 1974, 1975). Both dihydrotestosterone and testosterone can be specifically bound in seminiferous tubules of immature rats (Hansson et al., 1974). From previous results it also appeared that binding of testosterone to the receptor in tubuli is mainly the result of binding in the Sertoli cells (Mulder et al., 1976). Several authors have presented results which were considered to reflect specific binding of androgens to germinal cells (Sanborn et al., 1975a,b; Galena et al., 1974). Only a limited physicochemical characterization of binding proteins in germinal cells has been performed and it remains to be shown that the apparent binding of androgens by germinal cells is caused by a protein

with properties similar to the properties of the receptor for androgens found in Sertoli cells. We have therefore tried to compare the specific nuclear binding of testosterone in Sertoli cell and germinal cell preparations obtained from rat testis tissue by extraction and separation of nuclear receptor–steroid complexes after in vitro and in vivo labelling with [^{3}H]testosterone.

MATERIALS AND METHODS

Animals

Immature male Wistar rats, substrain R-Amsterdam, were used in this study. Intact 30–35-day-old rats were used for experiments in which isolated cells were labelled with [^{3}H]testosterone in vitro. Other 30–35-day-old rats were hypophysectomized under ether anesthesia with a Hoffmann–Reiter hypophysectomy instrument (Neuman & Co., Ill., U.S.A.) and used 2 days after hypophysectomy for experiments in which [^{3}H]testosterone was administered in vivo.

Preparation of isolated germinal cells and Sertoli cell aggregates

The testes of 8 rats were decapsulated, chopped and treated with trypsin (0.1%, Code TRL; Worthington Biochemical Co., Freehold, N.J., U.S.A.) and deoxyribonuclease (20 μg/ml, DN-25; Sigma Chemical Co., St. Louis, Mo., U.S.A.) for 30 min at room temperature as described previously (Grootegoed et al., 1977). After addition of soybean trypsin inhibitor (0.1%, I-S; Sigma) and bovine serum albumin (0.4%, fraction V, B grade; Calbiochem., San Diego, Calif., U.S.A.) clumps of non-dissociated tissue were removed with a sieve (mesh width 2 mm) and a mixture of dissociated cells and tubular pieces was obtained. The dissociated cells were purified by sedimentation at unit gravity in an albumin gradient as described by Grootegoed et al. (1977). Sedimentation was performed at room temperature (22°C) for labelling in vitro and at 4°C after labelling in vivo of testicular tissue. After 80 and 100 min of sedimentation at room temperature and 4°C respectively, the fractions enriched in spermatocytes and round spermatids were collected and pooled (see table 1 for composition of pooled fractions) and suspended in 3 ml of medium A (essentially Eagle's Minimum Essential Medium but with 69.8 mM NaCl and 56.9 mM KCl, supplemented with 0.4% albumin).

The tubule fragments which were not digested during the trypsin treatment were washed and treated with collagenase and dispersed into cell aggregates as described by Fritz et al. (1976). By this procedure the peritubular cells and a part of the germinal cells were removed and a preparation enriched in Sertoli cells was obtained. This preparation was suspended in 3–6 ml of medium A. In one experiment a part of the Sertoli cell aggregates was treated for 10 min with trypsin (0.1%) and deoxyribonuclease (20 μg/ml). After addition of trypsin inhibitor and washing, the cell aggregates were suspended in 3 ml of medium A. The Sertoli cell aggregates were left at room temperature or at 4°C in medium A until the purification of the

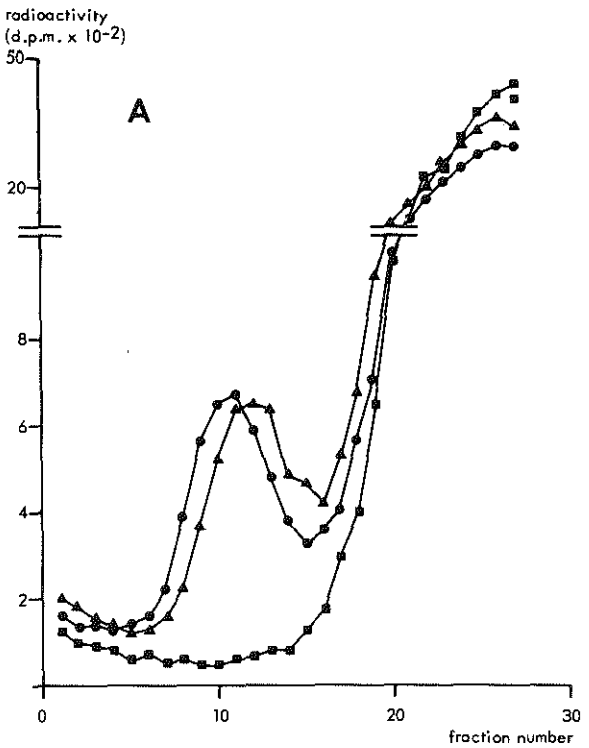
germinal cells at the corresponding temperatures was completed. Testosterone binding activity was estimated in the germinal cell and Sertoli cell preparations 4 h after killing of the rats.

Estimation of testosterone binding

For in vitro labelling the germinal cells and Sertoli cells (about 6–10 mg of protein), isolated from intact rats, were incubated in 3 ml of medium A with 2×10^{-8} M [1,2,6,7-^{3}H]testosterone (spec. act. 84 Ci/mmol) for 60 min at 32°C. In control experiments 2×10^{-6} M nonradioactive testosterone was also added to the incubation medium. For in vivo labelling 5 μCi of [1,2,6,7-^{3}H]testosterone (spec. act. 84 Ci/mmol) in 20 μl of Hank's Balanced Salt Solution was injected in each testis of hypophysectomized rats. After 30–60 min the rats were killed and the testes used for the isolation of germinal cell and Sertoli cell preparations. The cell preparations, labelled in vitro or in vivo, were homogenized and the nuclear pellets were washed with Triton X-100 and extracted with 0.4 M KCl as described by Mulder et al. (1976). The subsequent separation of free and bound steroid in the KCl extract by sucrose density gradient centrifugation in sucrose gradients containing 0.4 M KCl, protein estimation and measurement of radioactivity were performed as described by de Boer et al. (1977).

RESULTS AND DISCUSSION

The presence of androgen receptors was examined in the nuclear fractions of preparations of Sertoli cells and germinal cells after incubation of the preparations with radioactive testosterone for 60 min at 32°C. Cytoplasmic androgen receptors, if present, can be expected to form a complex with the radioactive testosterone and to be translocated subsequently to nuclear acceptor sites during the incubation period (King and Mainwaring, 1974). Sucrose gradient centrifugation of the 0.4 M KCl extract of the nuclear fraction of [^{3}H]testosterone labelled Sertoli cells gave a peak of radioactive material sedimenting around fractions 8–14 (the 4–5S region of the gradient) (fig. 1A). This peak was absent if unlabelled testosterone (2×10^{-6} M) in addition to the [^{3}H]testosterone was added to the incubation medium (fig. 1A); it was concluded therefore that the peak in fractions 8–14 represented binding of [^{3}H]testosterone to proteins with limited binding capacity. The amount of [^{3}H]testosterone bound was estimated to correspond with $53–115 \times 10^{-15}$ mol binding sites per mg of protein in the 0.4 M KCl extract (table 1). The results are in agreement with previous observations of Mulder et al. (1976). These authors have further characterized the androgen binding in testicular tissue with respect to steroid specificity, dissociation rate at 4°C and thermolability and they have shown that the observed binding of androgens was binding to a receptor protein and not to testicular androgen binding protein (ABP) (Mulder et al., 1975). When the KCl extract of the nuclear fraction of the [^{3}H]testosterone labelled germinal cells was



subjected to sucrose gradient centrifugation, no distinct peak but only a small elevation was present in the radioactive profile around fractions 12–14 (fig. 1B). The small amount of labelled androgen possibly bound to nuclear proteins of the germinal cell preparation may be partly explained by the presence of approximately 2% of Sertoli cells (table 1).

The presence of endogenous testosterone could cause an underestimation of the number of active binding sites. The endogenous testosterone concentration in the isolated germinal cells, however, was less than 0.7 pmol/6 mg of protein (estimated by radioimmunoassay as described by Verjans et al., 1973), which is about 100 times lower than the amount of radioactive steroid present during the incubation. Extensive washing of the cells during the sedimentation could have contributed to the low level of testosterone. Moreover, germinal cells and Sertoli cell aggregates were isolated from the same testicular tissue and a large number of nuclear androgen binding sites were estimated in the Sertoli cell aggregates, indicating that the receptor assay was not markedly disturbed by the presence of traces of endogenous testosterone.

It was shown previously that spermatocytes, isolated with the same procedure

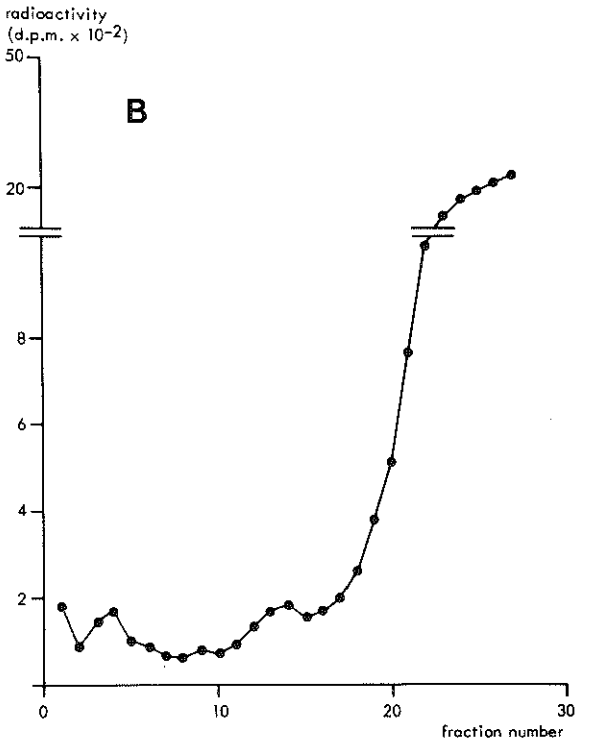


Fig. 1. Gradient centrifugation of the KCl extracts of testicular nuclei. Isolated cells were incubated for 60 min at 32°C with 2×10^{-8} M [^{3}H] testosterone. A 200 µl portion of the nuclear extracts (240–340 µg of protein) was fractionated. (A) ●———●, Sertoli cell aggregates; ■———■, Sertoli cell aggregates incubated with [^{3}H] testosterone and 2×10^{-6} M unlabelled testosterone; ▲———▲, Sertoli cell aggregates treated with trypsin (0.1%) for 10 min and subsequently incubated with [^{3}H] testosterone. (B) ●———●, germinal cells.

as used in the present experiments, were intact with respect to the synthesis of different species of RNA (Grootegoed et al., 1977). The germinal cells might, however, lose the ability to form functional steroid–receptor complexes during the treatment of the testicular tissue with trypsin. Compared to the germinal cells, the Sertoli cells were probably less exposed to trypsin since the peritubular cells and germinal cells surrounded the Sertoli cells when the testicular tissue was initially treated with trypsin. In one experiment we therefore treated isolated Sertoli cell aggregates (peritubular cells removed) with 0.1% trypsin for 10 min before the cells were labelled with [^{3}H] testosterone and it appeared that the binding activity was not changed after this treatment (fig. 1A, table 1). The estimation of cellular steroid receptor contents in the present experiments may therefore not have been disturbed by the use of trypsin.

Table 1
Binding of [^{3}H]testosterone in the KCl extract of testicular nuclei

Preparation	Amount of testosterone bound (10^{-15} mol binding sites/mg of protein in nuclear extract)		Cellular composition (% of cell types present)		
			Sertoli cells	Germinal cells	Other cell types
	in vitro	in vivo			
Germinal cells	3; 11	0; 0	<1–2	90–96 [a]	2–8
Sertoli cell aggregates	53; 115	53; 91	40–50	50–60	5
Sertoli cell aggregates treated with trypsin	69				

Testicular cells were labelled with [^{3}H]testosterone in vitro and in vivo and the number of binding sites occupied with [^{3}H]testosterone in the 0.4 M KCl extracts of the nuclear fractions was estimated. Bound and unbound steroids were separated by centrifugation of the extracts in sucrose gradients (see also figs. 1 and 2). Samples of the Sertoli cell preparations were fixed in Bouin's fixative, sections were made and the cellular composition was estimated by counting nuclei. The germinal cells were fixed in Bouin's fixative on microscope slides, stained with hematoxylin and eosin and identified as described by Platz et al. (1975). In each germinal cell preparation 600 *nuclei* were counted.

[a] Spermatogonia and preleptotene spermatocytes 4 ± 2, spermatocytes 30 ± 10, round spermatids 58 ± 12, elongating spermatids 2 ± 2 (mean percentage of 4 experiments ±SD).

Cytoplasmic receptors for androgens in isolated germinal cells may be very labile. If the binding sites are occupied in vivo by radioactive testosterone and translocated to the nucleus they may be well protected during the isolation procedure. We therefore injected [^{3}H]testosterone in vivo, in testes of rats which were used 2 days after hypophysectomy to lower the level of endogenous testosterone. The results of these experiments, presented in fig. 2 and table 1, were similar to the results obtained after labelling with [^{3}H]testosterone in vitro, and also indicate the presence of nuclear binding sites for androgens in the Sertoli cell aggregates but not in the preparations of germinal cells.

We have concluded from the present results that a germinal cell preparation, containing mainly primary spermatocytes and early spermatids, probably does not bind testosterone to a nuclear receptor. Sanborn et al. (1975a,b) have obtained labelling of nuclei of germinal cells from rat testis after a nuclear exchange assay with [^{3}H]testosterone and the exchange activity per mg of DNA was highest in late spermatids, sperm and sperm heads. These observations are not necessarily in disagreement with the present results since the testis of the immature rats used in the present experiments did not contain late spermatids. The specificity of the binding shown by nuclear exchange seems, however, questionable as long as no

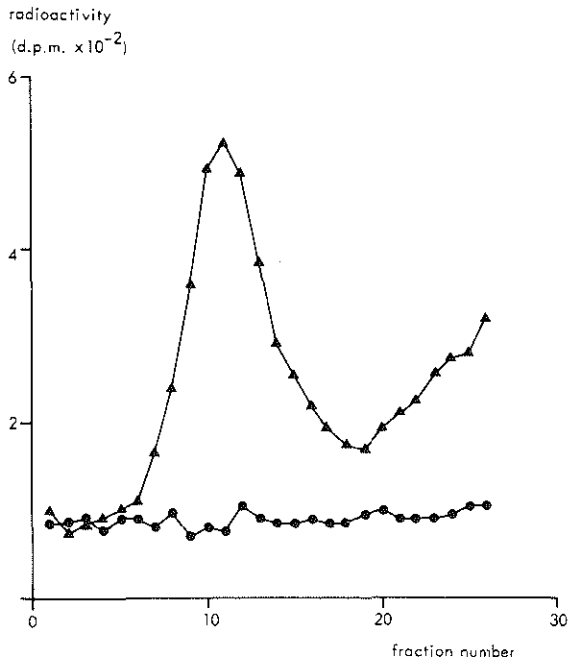


Fig. 2. Gradient centrifugation of the KCl extracts of testicular nuclei. Testicular tissue of hypophysectomized rats was labelled in vivo (5 μCi of [^{3}H]testosterone injected per testis) for 30–60 min. A 200 μl portion of the nuclear extracts (120–140 μg of protein) was fractionated. ▲———▲, Sertoli cell aggregates; ●———●, germinal cells.

separation of the nuclear receptor–steroid complex by sucrose gradient centrifugation or other separation techniques has been performed. Sanborn et al. (1975a,b) have concluded that it was not possible on the basis of the evidence obtained "to equate nuclear exchange activity in the testis with the presence of nuclear-bound receptor". Galena et al. (1974) have suggested the presence of progesterone and androgen receptors in nonflagellate germ cells of rat testis. It is possible, however, that in their study the steroids were not bound to receptors since almost no radioactivity was found in the nuclear fraction after incubation of the isolated cells with labelled steroid for 30 min at 37°C. Also, the steroid–protein complexes were shown to be stable at 50°C, whereas testicular androgen–receptor complexes appear to dissociate at this temperature (Hansson et al., 1974; Mulder et al., 1975). Tsai et al. (1977) observed the presence of chromatin acceptor sites for testicular receptor–androgen complex in purified preparations of Sertoli cells and testicular germ cells, and these authors therefore suggested that both cell types are primary targets for androgens. The binding observed, however, does not necessarily indicate that the germinal cells contain androgen receptors.

From the present results it cannot be excluded that germinal cells bind testosterone to a nuclear receptor during a very restricted stage of their development and that binding activity is lost at a successive stage and therefore cannot be detected in germinal cell preparations which contain several stages. Alternatively, it is more probable that testosterone is not bound to nuclear receptors in germinal cells, but is bound only to the nuclear receptors in Sertoli cells, and that the Sertoli cells are mediators of the influences of testosterone on germinal cells. The last suggestion is supported by results from experiments with male mice chimeric for androgen-resistant (Tfm/y) and normal (+/y) genotype (Lyon et al., 1975). The Tfm locus, located on the X-chromosome, controls a cytoplasmic androgen receptor which is absent in Tfm/y cells. In male mice of the genotype Tfm/y spermatogenesis is arrested at the first meiotic division but the chimeric mice produced offspring from their Tfm/y component. The testes of the chimeric mice will contain +/y Sertoli cells and Tfm/y Sertoli cells and the +/y Sertoli cells may enable the production of Tfm/y spermatozoa (Lyon et al., 1975).

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REFERENCES

Clermont, Y. and Harvey, S.C. (1967) Ciba Found. Colloq. Endocrinol. 16, 173.

de Boer, W., de Vries, J., Mulder, E. and van der Molen, H.J. (1977) Biochem. J. 162, 331.

Fritz, I.B., Rommerts, F.F.G., Louis, B.G. and Dorrington, J.H. (1976) J. Reprod. Fertil. 46, 17.

Galena, H.J., Pillai, A.K. and Terner, C. (1974) J. Endocrinol. 63, 223.

Grootegoed, J.A., Grollé-Hey, A.H., Rommerts, F.F.G. and van der Molen, J.H. (1977) accepted for publication in Biochem. J.

Hansson, V., McLean, W.S., Smith, A.A., Tindal, D.J., Weddington, S.C., Nayfeh, S.N., French, F.S. and Ritzén, E.M. (1974) Steroids 23, 823.

King, R.J.B. and Mainwaring, W.I.P. (1974) Steroid–Cell Interactions (Butterworths, London).

Lyon, M.F., Glenister, P.H. and Lamoreux, M.L. (1975) Nature (London) 258, 620.

Mulder, E., Peters, M.J., van Beurden, W.M.O. and van der Molen, H.J. (1974) FEBS Lett. 47, 209.

Mulder, E., Peters, M.J., de Vries, J. and van der Molen, H.J. (1975) Mol. Cell. Endocrinol. 2, 171.

Mulder, E., Peters, M.J., van Beurden, W.M.O., Galdieri, M., Rommerts, F.F.G., Janszen, F.H.A. and van der Molen, H.J. (1976) J. Endocrinol. 70, 331.

Platz, R.D., Grimes, S.R., Meistrich, M.L. and Hnilica, L.S. (1975) J. Biol. Chem. 250, 5791.

Sanborn, B.M. and Steinberger, E. (1975a) Endocrinol. Res. Commun. 2, 335.

Sanborn, B.M., Steinberger, A., Meistrich, M.L. and Steinberger, E. (1975b) J. Steroid Biochem. 6, 1459.

Tsai, Y.-H., Sanborn, B.M., Steinberger, A. and Steinberger, E. (1977) Biochem. Biophys. Res. Commun. 75, 366.

Verjans, H.L., Cooke, B.A., de Jong, F.H., de Jong, C.C.M. and van der Molen, H.J. (1973) J. Steroid Biochem. 4, 665.

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Ribonucleic Acid Synthesis *in vitro* in Primary Spermatocytes Isolated from Rat Testis

By J. ANTON GROOTEGOED, ANNE H. GROLLÉ-HEY, FOCKO F. G. ROMMERTS and HENK J. VAN DER MOLEN
Department of Biochemistry (Division of Chemical Endocrinology), Medical Faculty, Erasmus University Rotterdam, Rotterdam, The Netherlands

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The incorporation of [^{3}H]uridine into RNA was studied quantitatively (by incorporation of [^{3}H]uridine into acid-precipitable material) and qualitatively (by phenol extraction and electrophoretic separation of RNA in polyacrylamide gels) in preparations enriched in primary spermatocytes, obtained from testes of rats 26 or 32 days old. The rate of incorporation of [^{3}H]uridine into RNA of isolated spermatocytes was constant during the first 8 h of incubation, after which it decreased, but the decreased rate of incorporation was not reflected in a marked change in electrophoretic profiles of labelled RNA. In isolated spermatocytes, [^{3}H]uridine was incorporated mainly into heterogeneous RNA with a low electrophoretic mobility. Most of this RNA was labile, as shown when further RNA synthesis was inhibited with actinomycin D. Spermatocytes *in vivo* also synthesized heterogeneous RNA with a low electrophoretic mobility. A low rate of incorporation of [^{3}H]-uridine into rRNA of isolated spermatocytes was observed. The cleavage of 32S precursor rRNA to 28S rRNA was probably retarded in spermatocytes *in vitro* as well as *in vivo*. RNA synthesis by preparations enriched in early spermatids or Sertoli cells was qualitatively different from RNA synthesis by the spermatocyte preparations. It is concluded that isolated primary spermatocytes maintain a specific pattern of RNA synthesis, which resembles RNA synthesis in spermatocytes *in vivo*. Therefore isolated spermatocytes of the rat can be used for studying the possible regulation of RNA synthesis during the meiotic prophase.

In rat testis, spermatocytes arise from diploid stem cells via mitotic divisions. The spermatocytes go through the meiotic divisions and give rise to haploid spermatids which after transformation result in the formation of spermatozoa. Several steps of spermatogenesis, including the prophase of meiosis in primary spermatocytes and the meiotic divisions, require the presence of testosterone and/or follitropin (Clermont & Harvey, 1967). The molecular mechanisms of action of testosterone and/or follitropin on primary spermatocytes are not known. RNA synthesis in the spermatocytes may be involved in these actions because the action of hormones on cells or tissues in many systems is mediated via an effect on RNA metabolism (Woo & O'Malley, 1975; Means, 1971).

RNA synthesis during meiosis in male mammals has been studied previously in the rat (Söderström & Parvinen, 1976), mouse (Monesi, 1965; Kierszenbaum & Tres, 1974*a*), hamster (Utakoji, 1966) and ram (Loir, 1972), by using radioautographic techniques and the biochemical analysis of total testicular tissue or seminiferous tubules. Transcription during the meiotic prophase appeared to be almost completely restricted to the autosomes (Kierszenbaum & Tres, 1974*a*), and it has been suggested that this transcription represented mainly the synthesis of heterogeneous nuclear RNA (Kierszenbaum & Tres, 1974*b*; Söderström & Parvinen, 1976). Radioautographic experiments have shown a low rate of RNA synthesis in nucleoli during meiotic prophase in the testis of mouse and rat (Kierszenbaum & Tres, 1974*a*; Stefanini *et al.*, 1974).

Biochemical analysis of RNA synthesis in total testicular tissue or seminiferous tubules, which contain many cell types, will give limited information about RNA synthesis in specific germinal cell types. We therefore decided to study RNA synthesis in isolated primary spermatocytes (pachytene stage, prophase of the first meiotic division) from rat testis, and the present results indicate that it is possible to analyse the metabolism of heterogeneous RNA and rRNA in such isolated germinal cells.

Materials and Methods

Animals

Male Wistar rats, substrain R-Amsterdam (26 days or, where indicated, 32 days old) were used in the

present study. In 26-day-old rats, spermatogenesis has reached the meiotic divisions and the first spermatids have formed. In testes from 32-day-old rats, spermatogenesis has progressed until stage 8, and a large number of early spermatids (steps 1–8) are present.

Chemicals

[5-^{3}H]Uridine (specific radioactivities 5 Ci/mmol and 30 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sodium tri-isopropylnaphthalenesulphonate was supplied by Eastman Organic Chemicals, Rochester, NY, U.S.A. Sodium 4-aminosalicylate and colourless phenol (AnalaR) were purchased from BDH, Poole, Dorset, U.K. *m*-Cresol was distilled before use to obtain a colourless preparation. Formamide was deionized by shaking for several hours with a mixed-bed resin (Bio-Rad AG 501-X8; Bio-Rad Laboratories, Richmond, CA, U.S.A.; formamide/resin, 19:1, v/w). Acrylamide and *NN'*-methylenebisacrylamide of electrophoresis purity were purchased from Bio-Rad. Other chemicals used were of highest available purity. All glassware was heat-sterilized. Solutions were made in glass-distilled and autoclaved water. Media used for isolation of cells were sterilized by ultrafiltration (filter 0.2 μm pore size).

Preparation of cell suspensions

Rats were killed by cervical dislocation; the testes were collected at room temperature (22°C) and the tunica albuginea was removed from the testes. The testes (1.2–1.6 g wet wt.) were chopped into pieces about 0.5 mm^3, and 20 ml of isolation medium [essentially Hanks balanced salt solution (Hanks & Wallace, 1949) but with 69.8 mM-NaCl and 85.8 mM-KCl, supplemented with penicillin (10^5 i.u./litre), streptomycin (100 mg/litre) and Fungizone (1 mg/litre; Gibco, Grand Island, NY, U.S.A.)] was added to the tissue pieces in a 100 ml flask. Subsequently DNAase* (DN-25; Sigma Chemical Co., St. Louis, MO, U.S.A.) and trypsin (Code TRL; Worthington Biochemical Corporation, Freehold, NJ, U.S.A.), dissolved in isolation medium, were added to give final concentrations of 20 μg/ml and 0.1%. After shaking gently for 30 min, bovine serum albumin (fraction V, B grade; Calbiochem, San Diego, CA, U.S.A.) (0.1 g dissolved in 5 ml of isolation medium), soya-bean trypsin inhibitor (type I-S; Sigma) (20 mg) and DNAase (DN-25) (200 μg) were added to the cell suspension. Residual tissue fragments were removed by filtration through eight layers of aseptic gauze, and 24 ml of the cell suspension (2×10^6 cells/ml) was immediately used for separation of the different cell types.

* Abbreviation: DNAase, deoxyribonuclease.

Isolation of primary spermatocytes

The cells were separated by using the 'Staput' method of velocity sedimentation at unit gravity (Miller & Phillips, 1969; Meistrich *et al.*, 1973). Cell separation was performed at room temperature or, when indicated, at 4°C. The Perspex column (diam. 12 cm) was filled from the bottom successively with 60 ml of isolation medium, 24 ml of the cell suspension, and a non-linear albumin gradient in isolation medium [1% (w/v) (40 ml); 2% (240 ml); 3.2% (240 ml)]. Loading of the column was performed at a rate of 15 ml/min. Spermatocytes in the pachytene stage had sedimented 22–26 mm within 80 min (at room temperature) or 100 min (at 4°C) after the start of the loading procedure. During these periods, round early spermatids sedimented 10–13 mm. The sedimentation column was emptied at a rate of 15 ml/min and the spermatocyte fraction was collected. In some experiments, when 32-day-old rats were used, the early spermatid fraction was also collected. The fractions were centrifuged for 6 min at 200*g* and the cell pellet was suspended in incubation medium [essentially Eagle's Minimum Essential Medium (Flow Laboratories Ltd., Irvine, Ayrshire, Scotland, U.K.), but with 69.8 mM-NaCl and 56.9 mM-KCl, supplemented with 1 mM-sodium pyruvate, 6 mM-sodium DL-lactate, bovine serum albumin (4 g/litre), penicillin (10^5 i.u./litre), streptomycin (100 mg/litre) and Fungizone (1 mg/litre)] to which pure DNAase (DN-CL; Sigma) (final concn. 2.0 μg/ml) was added.

After a second centrifugation for 4 min at 200*g* the cells were resuspended in incubation medium and 2 ml portions of the cell suspension (0.5×10^5–2×10^5 cells/ml) were incubated in a plastic Petri dish (diam. 3 cm) at 32°C in an atmosphere of air/CO_2 (19:1). The small amount of DNAase (0.05–0.1 μg/ml) that was still present after the washing procedure was necessary to prevent excessive cell clumping during the incubation.

The whole procedure from death of the rats until the incubation of the cells was completed within 3 h. Trypan Blue was excluded by 95% or more of the cells. The composition of the 'Staput' fractions was evaluated by microscopy of air-dried preparations fixed in Bouin's fluid and stained with Haematoxylin and Eosin.

Qualitative analysis of RNA synthesis

Cells (3×10^5–4×10^5) were preincubated for 2 h or longer, and RNA labelling was started by the addition of 10–50 μCi of [5-^{3}H]uridine/ml (specific radioactivity 30 Ci/mmol). Incubation was stopped after 1–20 h of labelling by washing the cells twice at

4°C with 0.9% NaCl supplemented with unlabelled 0.5mM-uridine, and the cells were frozen in solid CO_2. RNA was extracted by a modification of the method described by Parish & Kirby (1966), as follows. RNA from *Escherichia coli* (BDH) was added as carrier (25–50μg). The cells were lysed at 0°C in 0.4ml of solution A [6% (w/v) sodium 4-aminosalicylate, 1% (w/v) sodium tri-isopropylnaphthalenesulphonate, 6% (v/v) butan-2-ol in water]. An equal volume of solution B (500ml of phenol, 70ml of *m*-cresol, 55ml of water and 0.5g of 8-hydroxyquinoline) was added and the mixture was shaken at room temperature for 10min. After centrifugation (10000rev./min, 10min at 0°C) the water layer was removed and kept. The remaining interphase and phenol phase were mixed for 5min with a second volume (0.2ml) of solution A and after centrifugation (10000rev./min, 10min at 0°C) the water layer was added to the first water layer. NaCl (final concn. 3%, w/v) was added to the combined water layers and a second deproteinization with 0.2ml of solution B was carried out. The nucleic acids in the water phase were precipitated by adding 2vol. of ethanol containing 10% (w/v) *m*-cresol and leaving the mixture for 45min at 0°C. The precipitate was washed twice with 0.2ml of 0.2M-sodium acetate in 75% (v/v) ethanol. DNA was removed by treatment of the sample with 50μg of ribonuclease-free DNAase/ml (code DPFF; Worthington) in 7mM-$MgCl_2$/100mM-Tris/HCl (pH7.5) for 30min at 0°C (Spradling *et al.*, 1974). RNA was precipitated by adding 2vol. of ethanol and leaving the mixture for 45min at 0°C. The precipitated RNA was washed successively with 0.2ml of 0.2M-sodium acetate in 75% ethanol and with 0.2ml of 96% ethanol. The RNA was dissolved in 50μl of 95% (v/v) formamide, containing 2mM-EDTA and 20mM-Tris/HCl (pH7.4). Before application to the gel the dissolved RNA was heated at 37°C for 10min (Slack *et al.*, 1975) and cooled in ice.

Polyacrylamide gels (2.4%), cross-linked with *NN'*-methylenebisacrylamide, were prepared as described by Bishop *et al.* (1967). After washing and swelling of the gels during 72h in buffer E (0.4M-Tris, 0.02M-sodium acetate, 0.001M-sodium EDTA adjusted to pH7.2 with acetic acid) (Bishop *et al.*, 1967) containing 0.2% sodium dodecyl sulphate, the gels were sucked into Plexiglass tubes (diam. 0.4cm) and cut at a length of 9cm. Buffer E containing 0.2% sodium dodecyl sulphate was used as electrophoresis buffer and the gels were pre-run for 30min at 3mA/gel and 70V. The gels were then loaded with 20–40μg of RNA dissolved in 40μl of 95% formamide, and electrophoresis was performed for 130min at 3mA/gel. To localize 28S and 18S rRNA (and *E. coli* 23S and 16S rRNA) in the gels after electrophoresis, the gels were scanned in water at 260nm with a Gilford spectrophotometer. *s* values of unknown peaks were calculated by the method of Lewicki & Sinskey (1970). The gels were subsequently frozen and cut into slices of 1.4mm. The slices were extracted in a glass liquid-scintillation-counting vial with 0.5ml of Soluene-350 (Packard Instrument S.A. Benelux, Brussels, Belgium) for 2h at 50°C, and the radioactivity in the extract was counted in toluene containing 4g of PPO (2,5-diphenyloxazole)/litre and 0.04g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre.

The amount of radioactivity in the gels was corrected for small differences in the amount of RNA applied to the gel, by using the A_{260} of 28S rRNA as a reference.

Quantitative analysis of RNA synthesis

Cells (1.0×10^5–1.2×10^5) were preincubated for 0, 2, 4, 6, 10 or 20h, after which they were incubated for 2h with [5-^{3}H]uridine (specific radioactivity 5Ci/mmol) (10μCi/ml). Incubation was stopped by the addition of ice-cold 0.9% NaCl containing 0.5mM-uridine. The cells were washed twice with 15ml of the 0.9% NaCl with 0.5mM-uridine and subsequently the cells were frozen and thawed twice. To each sample 0.5mg of RNA (type IV; Sigma) was added as a carrier and macromolecules were precipitated with 0.5ml of 1.0M-$HClO_4$ at 0°C. After centrifugation (2000rev./min; 5min) the supernatant was removed and kept, and the pellet was washed twice with 0.5ml of 1.0M-$HClO_4$. The supernatant and the washes were pooled for measurement of the amount of $HClO_4$-soluble radioactivity inside the cells. The pellet was washed with 0.5ml of 96% ethanol, and RNA was hydrolysed in 0.5ml of 1.0M-NaOH at 37°C for 60min. DNA and protein were precipitated by addition of 0.5ml of 3.0M-$HClO_4$ at 0°C. After centrifugation (2000rev./min; 5min) the supernatant was used for measurement of the amount of radioactivity incorporated into RNA ($HClO_4$-insoluble radioactivity). The pellet was washed with 96% ethanol, dissolved in 50μl of 0.4M-NH_3 at 0°C for 20h and the amount of DNA was measured by using a fluorimetric assay (Robertson & Tait, 1971). Radioactivity present in $HClO_4$-insoluble and -soluble material (reflecting respectively [^{3}H]uridine incorporated into RNA and [^{3}H]uridine taken up by the cells but not incorporated into RNA) was counted in Instagel (Packard) and expressed as d.p.m./μg of DNA.

Results

Maintenance of isolated spermatocytes

Media for the isolation and incubation of spermatocytes from rat testis contained a high concentration of K^+ (85.8 and 56.9mM respectively), comparable with that in the tubular fluid (50–112mM) that surrounds the spermatocytes *in vivo* (Tuck *et al.*, 1970). An

Table 1. *Isolation of germinal cells from rat testicular tissue*

Cell suspensions were prepared from total testicular tissue of the rat (see the Materials and Methods section) and the suspensions were fractionated by sedimentation at unit gravity (Meistrich *et al.*, 1973). The percentages of cell types present in the fractions collected from the sedimentation column are presented in this Table. Fractions A_1 and A_2, enriched in primary spermatocytes, sedimented over a distance of 22–26 mm during 80 min at room temperature or 110 min at 4°C respectively. Fraction B, containing mainly early spermatids, sedimented 10–13 mm during 110 min at 4°C. Fraction A_1 was obtained from 26-day-old rats, to minimize the contamination by (multinucleated) spermatids in this fraction; 32-day-old rats were used to obtain fractions A_2 and B, to prevent contamination by late spermatids in both fractions. Drops of the fractions were put on microscope slides, fixed in Bouin's fluid and stained with Haematoxylin and Eosin. The cells were identified by criteria developed for isolated cells from mouse testis (Meistrich *et al.*, 1973) as adapted for rat testicular cells (Platz *et al.*, 1975). Cells with more than one nucleus were counted as single cells. At least 600 cells were counted in each fraction.

	Cell types (%)		
Fraction ...	A_1	A_2	B
Age of rats (days) ...	26	32	32
Cell type			
Spermatogonia and preleptotene spermatocytes	3	2	2
Leptotene, zygotene and early-pachytene spermatocytes	3	2	1
Middle- and late-pachytene spermatocytes	80	77	1
Dividing cells and secondary spermatocytes	2	3	1
Early spermatids (steps 1–8)	5	10	89
Somatic cell types (including Leydig cells and Sertoli cells)	4	2	3
Unknown and broken cells	2	3	2
Cytoplasmic fragments	1	1	1

impression about the composition of the cell preparations from 26-day-old rats can be obtained from the results in Table 1 (Fraction A_1). The gross morphology of the isolated spermatocytes, evaluated by phase-contrast microscopy, remained intact for at least 20 h after isolation. The quantitative incorporation of [^{3}H]uridine into RNA of spermatocytes was constant during the first 8 h of incubation, but incorporation decreased after 10–20 h of incubation (Fig. 1). The ratio of the amount of [^{3}H]uridine incorporated into RNA to the amount of [^{3}H]uridine taken up by the cells was relatively constant during the whole incubation period of 20 h (Fig. 1). The results in Fig. 1 indicate that the uptake of [^{3}H]uridine by the cell

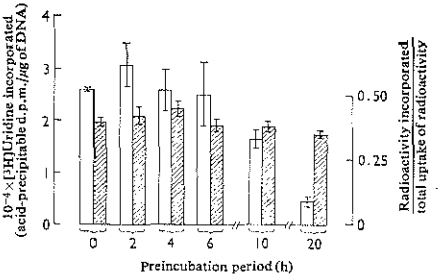

Fig. 1. *Uptake of [^{3}H]uridine by isolated spermatocytes and incorporation into RNA*

Isolated spermatocytes were preincubated for different time periods. After preincubation the incubation was continued in the presence of [^{3}H]uridine (10 μCi/ml, specific radioactivity 5 Ci/mmol) for 2 h. The amount of [^{3}H]uridine incorporated into RNA (□) and the ratio of the amount of [^{3}H]uridine incorporated into RNA to the total uptake of [^{3}H]-uridine (d.p.m in $HClO_4$-precipitable material/d.p.m. in $HClO_4$-precipitable plus $HClO_4$-soluble material) (▩) are presented. Means and range of individual mean values of two experiments are given. In each experiment the determinations were made in duplicate or in triplicate.

population decreased during the incubation period. The similarity of the two electrophoretic profiles (in Fig. 2) of RNA labelled during 8 h of incubation of the cells with [^{3}H]uridine after either 4 or 12 h of preincubation illustrates the absence of marked qualitative changes in the pattern of RNA synthesis after prolonged incubation of the cells.

Time sequence of RNA labelling

Within the first hour after addition of [^{3}H]uridine to isolated spermatocytes the label was incorporated into RNA with a heterogeneous electrophoretic mobility ('heterogeneous RNA'). Most of this labelled RNA moved more slowly than 28S rRNA, forming two peaks (calculated *s* value 50–55S and higher) (Fig. 3*a*). After 2 h of incubation in the presence of [^{3}H]uridine, an additional labelled component (calculated *s* value 40–45S) was present (Fig. 3*b*), which probably represented 45S rRNA, the precursor of 28S and 18S rRNA (Penman, 1966). Labelled 18S rRNA was detected 4 h after addition of [^{3}H]uridine. At the same time a radioactive component emerged with an electrophoretic mobility (calculated *s* value 32S) slightly less than that of 28S rRNA (Fig. 3*c*); this 32S RNA might represent the

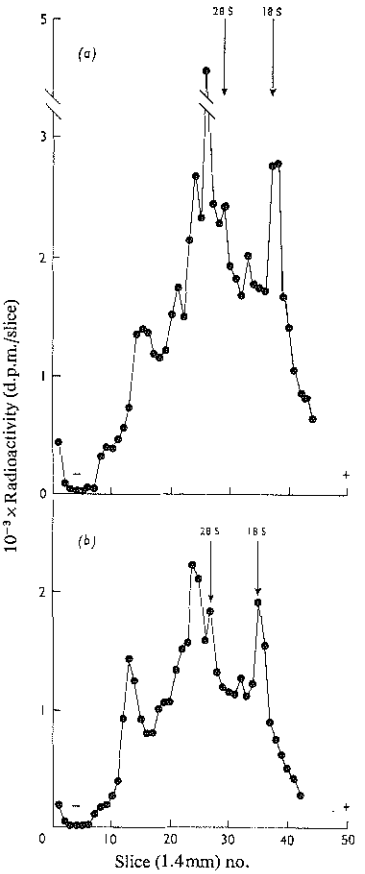

Fig. 2. *Effect of the preincubation period on qualitative RNA synthesis in isolated spermatocytes*
Spermatocytes were incubated *in vitro* with [^{3}H]-uridine (25μCi/ml) for 8h after preincubation in the absence of labelled precursor for 4h (*a*) or 12h (*b*). RNA was extracted, and was separated by electrophoresis in polyacrylamide gels as described in the text.

immediate precursor of 28S rRNA (Penman, 1966). Within 20h of incubation in the presence of [^{3}H]-uridine the specific radioactivity of 28S rRNA was increased relative to that of 32S RNA (Fig. 3*d*). The results in Fig. 3 show an accumulation of labelled 32S RNA, 4h and 20h after addition of [^{3}H]uridine to the isolated spermatocytes, but the amount of 32S RNA extracted from the spermatocytes (as determined from A_{260}) was small (Fig. 4).

Stability of heterogeneous RNA

After longer incubations of spermatocytes with [^{3}H]uridine the contribution of the slow-moving heterogeneous RNA to the profile of labelled RNA decreased relative to the amount of labelled 32S, 28S and 18S RNA, indicating that the heterogeneous RNA was not stable (Fig. 3). Moreover, no significant amount of the heterogeneous RNA was extracted from the spermatocytes, as determined from the A_{260} (Fig. 4). 'Chase' experiments were performed to investigate further the stability of the heterogeneous RNA synthesized *in vitro*. Because a 'chase' by isotope dilution was not sufficient to inhibit completely the incorporation of [^{3}H]uridine within a short time, actinomycin D was used for rapid inhibition of RNA synthesis (Perry & Kelley, 1970). Triplicate incubations of spermatocytes were labelled with [^{3}H]uridine for 1h. Subsequently the cells from one incubation were frozen for RNA extraction (Fig. 5*a*) and actinomycin D (final concn. 10μg/ml) was added to the cells in the other two incubations, and these cells were frozen for RNA extraction after 1h and 4h (Figs. 5*b* and 5*c*). After 4h in the presence of actinomycin D (Fig. 5*c*) the electrophoretic profile of labelled RNA showed a heterogeneous background and peaks of 32S and 18S rRNA. It is possible that part of the background activity represents non-ribosomal stable RNA. Most of the heterogeneous RNA synthesized during a short incubation period (Fig. 5*a*), however, disappeared in the presence of actinomycin D and was not converted into smaller and stable RNA molecules (Figs. 5*b* and 5*c*).

RNA synthesis in spermatocytes in vivo

In addition to the analysis of RNA synthesis in spermatocytes *in vitro*, we tried to label the RNA of the spermatocytes *in vivo*. [^{3}H]Uridine was injected into the testes of 32-day-old rats, and 4h later a cell suspension was prepared at room temperature from these testes. Subsequently the spermatocytes were isolated at 4°C (Fraction A_2 in Table 1) and RNA was extracted.

The electrophoretic profile of the RNA labelled *in vivo* (Fig. 6*a*) was similar to the profile obtained after 4h of labelling *in vitro* (Fig. 3*c*) with respect to the high rate of synthesis of heterogeneous RNA and the accumulation of labelled 32S RNA. Some turnover and processing of RNA may have occurred during the isolation of the cells after the labelling *in vivo*.

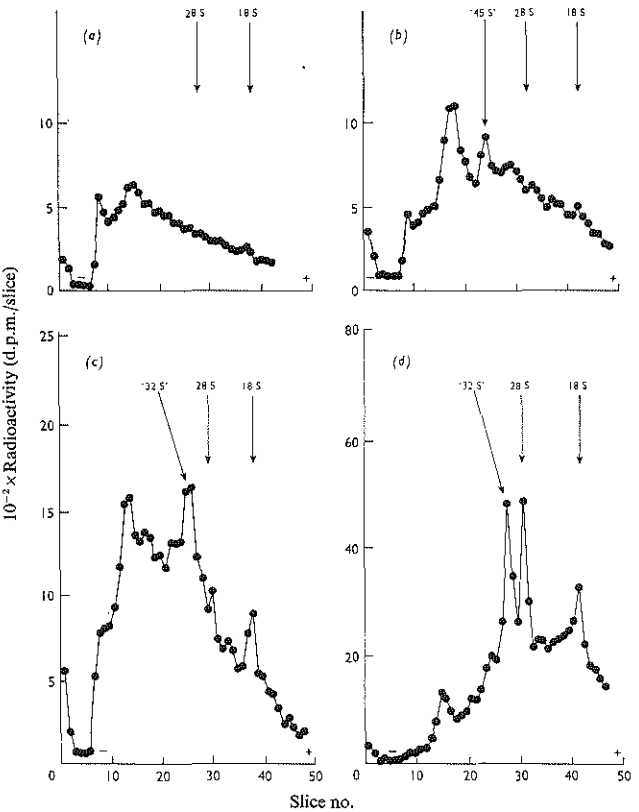

Fig. 3. *Time sequence of RNA labelling in isolated spermatocytes*
Spermatocytes were labelled *in vitro* with [^{3}H]uridine (*a*, *b* and *c* with 50 μCi/ml, *d* with 10 μCi/ml) and incubation was stopped after 1 h (*a*), 2 h (*b*), 4 h (*c*) or 20 h (*d*) of labelling. RNA was extracted and separated by electrophoresis in polyacrylamide gels. The labelled RNA peaks with a calculated *s* value of 32 S and 40–45 S are indicated as '32 S' and '45 S' respectively.

RNA synthesis in spermatids and Sertoli cells

To illustrate the specific features of RNA synthesis in spermatocytes, other testicular cell types were also labelled with [^{3}H]uridine, and the electrophoretic profiles of the labelled RNA were compared with those of the spermatocytes. A fraction enriched with early spermatids was collected from the sedimentation column (Fraction B in Table 1) after labelling testicular cells *in vivo*, by using 32-day-old rats. RNA was extracted from this spermatid fraction (Fig. 6*b*) as well as from Sertoli cells, which were labelled with [^{3}H]uridine in a culture system as described by Fritz *et al.* (1976) (Fig. 7). The electrophoretic profile of labelled RNA from the preparation of early spermatids showed the presence of labelled heterogeneous RNA with an electrophoretic mobility similar to that of heterogeneous RNA synthesized in spermatocytes. In Sertoli cells the labelled heterogeneous RNA with a low electrophoretic mobility contributed little to the total of labelled RNA; labelled heterogeneous RNA from Sertoli cells moved more rapidly during electrophoresis (around 18 S) than did the heterogeneous RNA extracted from the

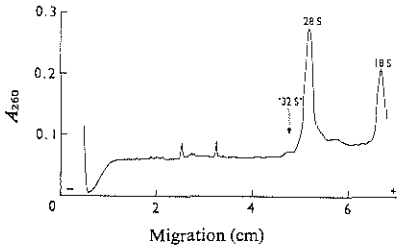

Fig. 4. *A_{260} of a polyacrylamide gel after electrophoresis of RNA extracted from spermatocytes*
RNA was extracted from isolated spermatocytes and separated by electrophoresis in a polyacrylamide gel (2.4%, w/v). The gel was scanned at 260nm after electrophoresis. The calculated position of '32S' RNA in the gel is indicated by an arrow.

germinal cells. In the preparation of early spermatids as well as in the cultured Sertoli cells, 45S rRNA was processed to 28S and 18S rRNA, and no marked accumulation of labelled 32S RNA occurred in these cell types.

Discussion

In isolated spermatocytes, the rate of [^{3}H]uridine incorporation was constant during 8h of incubation (Fig. 1). The observed decrease in the rate of incorporation after longer incubation periods was not accompanied by a change in the electrophoretic profile of labelled RNA (Fig. 2). Therefore the results suggest that, with respect to RNA metabolism, degeneration of isolated spermatocytes is reflected mainly in a decrease of the uptake of [^{3}H]uridine. We conclude that the analysis of RNA synthesis in isolated spermatocytes, under the conditions described, can be performed during at least 8h after isolation of the cells (the period of 3h required for isolation of the cells not included). The electrophoretic profiles of labelled RNA synthesized in spermatocytes *in vitro* (Fig. 3*c*) and *in vivo* (Fig. 6*a*) were similar, indicating that the synthesis of the different RNA species was not markedly altered after isolation. Moreover, RNA synthesis in spermatocytes was qualititatively different from RNA synthesis in preparations of early spermatids (Fig. 6*b*) and in cultured Sertoli cells (Fig. 7).

The present observations made on isolated spermatocytes show that the rate of synthesis of a heterogeneous class of RNA is much higher than the rate of synthesis of rRNA (Fig. 3), but the amount of slow-moving heterogeneous RNA extracted from spermatocytes was small (Fig. 4). It is concluded from the

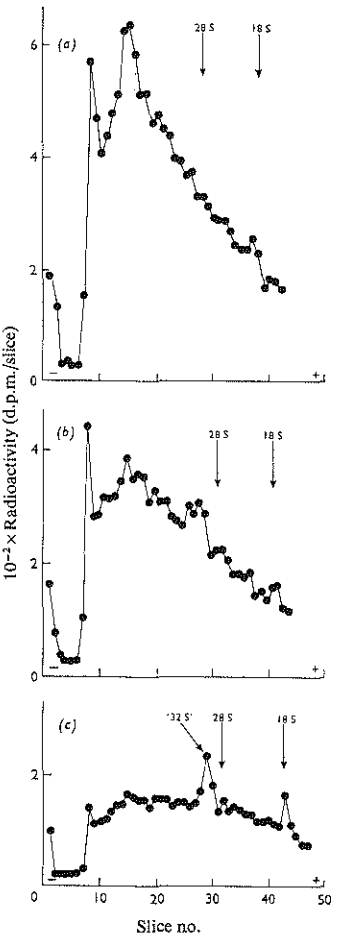

Fig. 5. *Stability of RNA in isolated spermatocytes during a 'chase' with actinomycin D*
Isolated spermatocytes were labelled with [^{3}H]-uridine (50μCi/ml) for 1h, after which actinomycin D (10μg/ml) was added to prevent further RNA synthesis. Incubation was stopped at 0h (*a*), 1h (*b*) or 4h (*c*) after addition of actinomycin D. RNA was extracted and separated by electrophoresis in polyacrylamide gels.

results in Fig. 5 that most of the heterogeneous RNA in spermatocytes is labile. The rate of decay of this RNA in the presence of actinomycin D (average

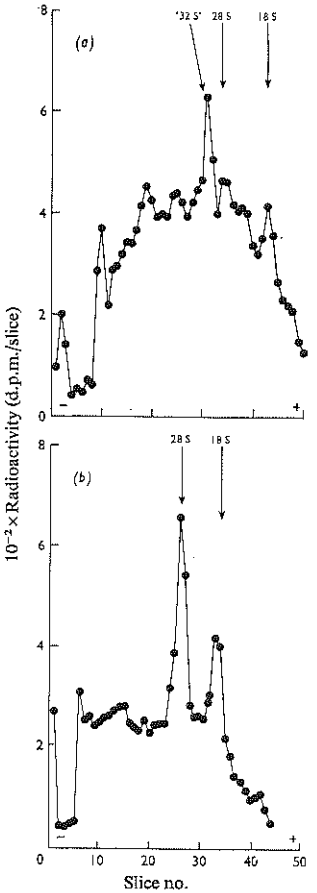

Fig. 6. *RNA synthesis in (a) spermatocytes and (b) early spermatids in vivo*
[^{3}H]Uridine was injected into the testes of 32-day-old rats (50 μCi/testis in Hanks balanced salt solution). After 4 h of labelling *in vivo*, fractions of primary spermatocytes and early spermatids were isolated at 4°C (Fractions A_2 and B in Table 1), and RNA was extracted and separated by electrophoresis in polyacrylamide gels. The amount of 28 S rRNA applied to the gels was five times the amount used for electrophoretic separation of RNA labelled *in vitro*.

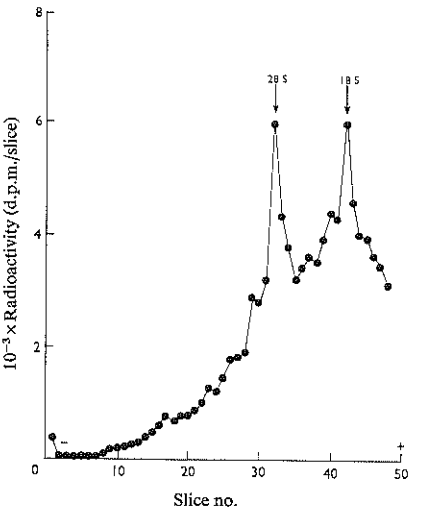

Fig. 7. *RNA synthesis in cultured Sertoli cells*
Electrophoretic separation in a polyacrylamide gel of ^{3}H-labelled RNA extracted from cultured Sertoli cells (Fritz *et al.*, 1976). The Sertoli cells were incubated with [^{3}H]uridine (25 μCi/ml) for 4 h.

half-life 2–3 h) is roughly comparable with that described for nuclear RNA from HeLa cells (Scherrer *et al.*, 1970) and the heterogeneous RNA in spermatocytes may resemble the heterogeneous nuclear RNA in somatic cells, although this has not been experimentally verified.

Evidence for a predominant synthesis of a heterogeneous class of RNA was also found, by biochemical methods, in total testis tissue of the hamster (Muramatsu *et al.*, 1968) and in seminiferous tubules of the rat (Söderström & Parvinen, 1976), and it was suggested that this synthetic activity originated from spermatocytes. Muramatsu *et al.* (1968) concluded that the heterogeneous 'rapidly labelled' RNA is not preserved as relatively stable mRNA or as rRNA, but is rapidly and completely degraded. In contrast, Söderström & Parvinen (1976) concluded that a considerable part of the RNA synthesized in the seminiferous tubules is heterogeneous nuclear RNA with a long lifetime (at least 36 h). They further suggested that the stable heterogeneous nuclear RNA, synthesized mainly in spermatocytes, includes precursors for very stable mRNA which might be translated during late spermiogenesis; in late spermatids the condensed genome is not transcribed, although proteins are synthesized (Monesi, 1965). The results of the present experiments, however, indicate that most of the heterogeneous RNA from spermatocytes has a short half-life and that only a

small portion of the heterogeneous RNA synthesized in spermatocytes may belong to a class of stable heterogeneous RNA (Fig. 5).

With respect to the transcription of ribosomal DNA, the present observations show that in spermatocytes the primary gene product, 45S rRNA, was probably cleaved into 32S and 18S RNA (Figs. 3 and 6*a*), both presumably rRNA. The subsequent cleavage of 32S to 28S rRNA was retarded, as concluded from the accumulation of labelled 32S rRNA in the electrophoretic profile of RNA extracted from the whole cell. In somatic cells, 28S and 18S rRNA leave the nucleolus and pass into the cytoplasm (Penman, 1966) and the results obtained with spermatocytes could indicate, therefore, that the labelled 32S rRNA is located in the nucleolus and that labelled 18S rRNA is included in small ribosomal subunits in the cytoplasm. The 32S rRNA was probably not stable and might have been processed to 28S rRNA, as indicated by the time sequence of RNA labelling (Fig. 3) and the absence of a significant amount of 32S rRNA from the A_{260} profile (Fig. 4). In cell preparations containing mainly early spermatids (Fig. 6*b*) and Sertoli cells (Fig. 7), no accumulation of labelled 32S rRNA was observed.

Biochemical analysis of testicular tissue of the mouse labelled *in vivo* by Galdieri & Monesi (1974) led them to suggest a possible accumulation of rRNA precursors in the nucleolus during the meiotic prophase, although the evidence obtained was indirect and difficult to interpret. Parchman & Lin (1972) observed little synthesis of rRNA in nucleoli of lily microsporocytes during the later stage of meiotic prophase, and 'an RNA species involved in the ribosomal pathway' accumulated in the nucleoli. These data from the literature in combination with the present findings on the synthesis of rRNA in spermatocytes may indicate that the maturation of rRNA can be retarded during the pachytene stage in male germ cells.

In summary, the present biochemical results on RNA synthesis in spermatocytes indicate that the isolation and incubation of spermatocytes can be used to obtain information about RNA synthesis in these cells. On the basis of such information it should be possible to investigate whether hormones have an effect at the level of RNA synthesis in primary spermatocytes.

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References

Bishop, D. H. L., Claybrook, J. R. & Spiegelman, S. (1967) *J. Mol. Biol.* **26**, 373–387
Clermont, Y. & Harvey, S. C. (1967) *Ciba Found. Colloq. Endocrinol. Proc.* **16**, 173–189
Fritz, I. B., Rommerts, F. F. G., Louis, B. G. & Dorrington, J. H. (1976) *J. Reprod. Fertil.* **46**, 17–24
Galdieri, M. & Monesi, V. (1974) *Exp. Cell Res.* **85**, 287–295
Hanks, J. H. & Wallace, R. E. (1949) *Proc. Soc. Exp. Biol. Med.* **71**, 196–200
Kierszenbaum, A. L. & Tres, L. L. (1974*a*) *J. Cell Biol.* **60**, 39–53
Kierszenbaum, A. L. & Tres, L. L. (1974*b*) *J. Cell Biol.* **63**, 923–935
Lewicki, P. P. & Sinskey, A. J. (1970) *Anal. Biochem.* **33**, 273–278
Loir, M. (1972) *Ann. Biol. Anim. Biochim. Biophys.* **12**, 203–219
Means, A. R. (1971) *Endocrinology* **89**, 981–989
Meistrich, M. L., Bruce, W. R. & Clermont, Y. (1973) *Exp. Cell Res.* **79**, 213–227
Miller, R. G. & Phillips, R. A. (1969) *J. Cell. Physiol.* **73**, 191–201
Monesi, V. (1965) *Exp. Cell Res.* **39**, 197–224
Muramatsu, M., Utakoji, T. & Sugano, H. (1968) *Exp. Cell Res.* **53**, 278–283
Parchman, L. G. & Lin, K.-C. (1972) *Nature (London) New Biol.* **239**, 235–237
Parish, J. H. & Kirby, K. S. (1966) *Biochim. Biophys. Acta* **129**, 554–562
Penman, S. (1966) *J. Mol. Biol.* **17**, 117–130
Perry, R. P. & Kelley, D. E. (1970) *J. Cell. Physiol.* **76**, 127–140
Platz, R. D., Grimes, S. R., Meistrich, M. L. & Hnilica, L. S. (1975) *J. Biol. Chem.* **250**, 5791–5800
Robertson, F. W. & Tait, K. (1971) *Anal. Biochem.* **41**, 477–481
Scherrer, K., Spohr, G., Granboulan, N., Morel, C., Grosclaude, J. & Chezzi, C. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 539–554
Slack, J. M., Sartirana, M.-L. & Loening, U. E. (1975) *Nature (London)* **253**, 282–284
Söderström, K.-O. & Parvinen, M. (1976) *Mol. Cell. Endocrinol.* **5**, 181–199
Spradling, A., Penman, S., Saveria Campo, M. & Bishop, J. O. (1974) *Cell* **3**, 23–30
Stefanini, M., de Martino, C., d'Agostino, A., Agrestini, A. & Monesi, V. (1974) *Exp. Cell Res.* **86**, 166–170
Tuck, R. R., Setchell, B. P., Waites, G. M. H. & Young, J. A. (1970) *Pflügers Arch.* **318**, 225–243
Utakoji, T. (1966) *Exp. Cell Res.* **42**, 585–596
Woo, S. L. C. & O'Malley, B. W. (1975) *Life Sci.* **17**, 1039–1048

Submitted for publication

RIBONUCLEIC ACID SYNTHESIS IN VITRO IN PRIMARY SPERMATOCYTES ISOLATED FROM TESTES OF INTACT AND HYPOPHYSECTOMIZED RATS

J.A. Grootegoed, L.M. van Meerkerk, F.F.G. Rommerts and H.J. van der Molen

Department of Biochemistry (Division of Chemical Endocrinology), Medical Faculty, Erasmus University Rotterdam, Rotterdam, The Netherlands

SUMMARY

In the present study RNA synthesis was examined in vitro in pachytene spermatocytes from immature intact rats and immature rats 2-4 days after hypophysectomy. The uptake and incorporation of ^{3}H-uridine in spermatocytes isolated from hypophysectomized rats was decreased as compared to the activity of spermatocytes from intact rats, but the relative rate of uridine incorporation was almost not affected. Also, no qualitative changes were observed in the synthesis of "heterogeneous" RNA and rRNA in spermatocytes. Radioautographic experiments revealed that 21-25% of the pachytene spermatocytes isolated from hypophysectomized rats was unlabelled after incubation with ^{3}H-uridine, whereas only 1-5% of the pachytene spermatocytes from intact rats was unlabelled.

Thus it appears that in the absence of the pituitary gland, RNA synthesis in many spermatocytes remains unchanged for at least several days, whereas an increased number of the spermatocytes

becomes inactive. It is suggested that hormones do not control spermatocytes with respect to RNA synthesis as analyzed in the present experiments, but that the hormones control, through an unknown mechanism, the number of spermatocytes with apparent normal RNA synthesis.

INTRODUCTION

Hypophysectomy of rats will result in degeneration of the seminiferous epithelium as reflected in a decreased number of different germinal cell types (Clermont & Morgentaler, 1955). Spermatogenesis in adult hypophysectomized rats is qualitatively maintained when testosterone is administered daily to the rats (Clermont & Harvey, 1967) and follitropin may potentiate the effects of testosterone, especially in immature rats (Courot, 1970). The effects of follitropin and testosterone on the germinal cells may be dependent on the activity of Sertoli cells, because Sertoli cells from the rat appear to be target cells for follitropin (Means, 1975) and androgens (Louis & Fritz, 1977). In contrast, receptors for androgens, which are believed to be obligatory for androgen action, could not be detected in germinal cells (Grootegoed, Peters, Mulder, Rommerts & Van der Molen, 1977b) and there is also no evidence for a direct action of follitropin on germinal cells. It is, however, unknown which biochemical mechanism(s) in the germinal cells may be influenced when hormones act on the seminiferous epithelium. We have therefore tried to study if the effect of hypophysectomy on the degeneration of germinal cells is reflected in changes in RNA synthesis in the germinal cells.

Russel & Clermont (1977) have shown that the degeneration of middle pachytene spermatocytes and step 7 and 19 spermatids is more sensitive to the absence of hormones than the degeneration of other ger-

minal cell types. Also, middle pachytene spermatocytes are highly active with respect to the incorporation of RNA precursors (Söderström & Parvinen, 1976). Therefore pachytene spermatocytes were used in the present study.

We have previously shown that isolated primary spermatocytes (pachytene stage of first meiotic division) actively incorporate ^{3}H-uridine into RNA (Grootegoed, Grollé-Hey, Rommerts & Van der Molen, 1977a). From these studies it was concluded that the isolated spermatocytes maintain a specific pattern of RNA synthesis, which resembles RNA synthesis of spermatocytes *in vivo*.

In the present study we have analyzed and compared qualitatively and quantitatively the incorporation *in vitro* of radioactively labelled uridine into RNA of spermatocytes isolated from testes of intact and hypophysectomized rats.

MATERIALS AND METHODS

Materials

Follitropin (ovine NIH-FSH-S12) was a gift from the National Institute of Health, Bethesda, Maryland, USA. (5-^{3}H)Uridine (specific activities 5 Ci/mmol and 30 Ci/mmol) and (2-^{14}C)uridine (specific activity 60 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks, U.K. All materials used for the analysis of RNA have been described previously (Grootegoed et al., 1977a).

Animals

Immature male Wistar rats, substrain R-Amsterdam, were used in this study. The pituitary of ether-anaesthetized rats was removed with a Hoffmann-Reiter hypophysectomy instrument (Neuman & Co., Ill., USA). Intact control rats

were also anaesthetized with ether. Follitropin (60 µg in 0.2 ml of 0.9% NaCl) and testosterone propionate (2 mg in 0.1 ml of arachidis oil) were injected subcutaneously twice daily starting within 4 hours after hypophysectomy. Intact control rats received 0.9% NaCl and oil only. To obtain cell preparations enriched in pachytene spermatocytes 26-29 day-old rats (intact or 48-64 h after hypophysectomy) were used, whereas 30-35 day-old rats (intact or 64 h after hypophysectomy) were used to isolate spermatocyte preparations as well as cell preparations enriched in round spermatids.

Isolation and incubation of cells

We have described in detail previously (Grootegoed et al., 1977a) the methods, the composition of the isolation medium, which is essentially Hanks Balanced Salt Solution, and the incubation medium, which is essentially Eagle's Minimum Essential Medium (both media modified with an increased amount of KCl), which were used for the treatment of testicular tissue with trypsin and DNAase*, for the separation of germinal cells by velocity sedimentation at unit gravity and for the incubation of these cells at 32°C.

The pooled testes from 3-6 rats were weighed and used for the preparation of a cell suspension with trypsin and DNAase at room temperature (22°C). After separation of the cells by velocity sedimentation at room temperature, fractions enriched in primary spermatocytes in pachytene stage (fraction A) and, if 30-35 day-old rats were used, in addition fractions enriched in round spermatids (fraction B) were collected. The sedimentation rate and the cellular composition of such fractions have been described pre-

*Abbreviation: DNAase, deoxyribonuclease

viously (Grootegoed et al., 1977a). The isolated cells were suspended in incubation medium ($1\text{-}4 \times 10^5$ cells/ml) and were preincubated at 32°C for 2 h followed by incubation with ^{3}H-uridine for quantitative biochemical analysis of RNA synthesis and for radioautography.

To prepare tubular fragments, the testes were chopped at a distance of 1 mm in two perpendicular directions, treated with trypsin and DNAase at room temperature and clumps of non-dissociated tissue were removed with a sieve (mesh width 2 mm). The tubular fragments were washed twice with 60 ml of isolation medium with DNAase (DN-25; Sigma Chemical Co., St. Louis, MO., USA) (final concentration 5 µg/ml), and once with 60 ml of incubation medium without DNAase. The tubular fragments were incubated in incubation medium (approximately 500 mg of tissue/10 ml) at 32°C with ^{3}H-uridine or ^{14}C-uridine. After incubation pachytene spermatocytes were isolated from these labelled tubular fragments as follows. The tubular fragments were washed with isolation medium, collected and chopped in pieces about 0.25 mm. The chopped tubules were used for the preparation of a cell suspension with trypsin and DNAase at room temperature as described above and after separation of the cells by velocity sedimentation at 5°C fractions enriched in primary spermatocytes (fraction A) were collected, frozen at -70°C and stored for subsequent qualitative analysis of labelled RNA.

Analysis of RNA synthesis

The quantitative incorporation of ^{3}H-uridine into RNA was estimated in preparations enriched in pachytene spermatocytes and round early spermatids respectively, obtained from 30-35 day-old rats. The cell preparations were preincubated for 2 h and then incubated for 2 h with (5-^{3}H)uridine with a specific radioactivity of 5 Ci/mmol at a concentration of 10 µCi/ml and the uptake of ^{3}H-uridine and

Table I. Composition of cell preparations.

Germinal cell preparations were obtained, by means of velocity sedimentation at unit gravity, from 30-35-day-old rats (intact, hypophysectomized or hypophysectomized and treated with follitropin and testosterone propionate as described in Materials and Methods). The cellular composition of fractions A and B was estimated using light microscopy of cells, fixed in Bouin's fixative on microscope slides and stained with haematoxylin and eosin. Cells were identified as described by Platz, Grimes, Meistrich & Hnilica (1975). For each group of experiments 1000 nuclei were counted and the number of nuclei from different cell types is presented as percentage of the total number of nuclei.

I - intact rats

H - hypophysectomized rats

H/FSH/TP - hypophysectomized rats treated with follitropin and testosterone propionate

fraction	treatment of the rats	sperma-tocytes	sperma-tids	other cell types
fraction A (pachytene spermatocytes)	I and H/FSH/TP	53	38	9
	H	39	48	13
contaminating cells in fraction A (spermatids plus other cell types = 100%)	I and H/FSH/TP		80	20
	H		79	21
fraction B (round spermatids)	I and H/FSH/TP	7	83	10
	H	7	79	14

incorporation of ^{3}H-uridine into acid-precipitable material were estimated and expressed as dpm/µg of DNA, as described previously (Grootegoed et al., 1977a).

It was observed that the purity of the spermatocyte preparations (fraction A) varied, depending on the hypophysectomy, and the values for the activity (uptake and incorporation of ^{3}H-uridine per µg of DNA) of fraction A should therefore be corrected for contamination by other cell types. This correction could be carried out when 30-35 day-old rats were used because the cellular composition of the preparations of round spermatids (fraction B) obtained from these rats was relatively constant and appeared to be comparable with the cellular composition of the contaminating cells in fraction A (Table 1). Values for the activity of fraction B were therefore used to correct the values for the activity of fraction A for differences in composition. For this the cells in fraction A were grouped according to their DNA content (spermatids, 1C; spermatogonia and somatic cell types, 2C; early and pachytene spermatocytes, 4C) and the amount of DNA present in pachytene spermatocytes was expressed as part of the total amount of DNA in fraction A (this value varied from 0.48 to 0.79). Corrected values for the activity of pachytene spermatocytes were then calculated with the formula $A = P.S + (1-P)B$, in which P is the amount of DNA present in pachytene spermatocytes expressed as part of the total amount of DNA in fraction A, A is the value for the activity of fraction A per µg of DNA, B is the value for the activity of fraction B per µg of DNA, S is the corrected value for the activity of pachytene spermatocytes.

The incorporation of ^{3}H- or ^{14}C-uridine into RNA of spermatocytes of 26-29 day-old rats was studied qualitatively after phenol extraction of total cellular RNA and electrophoretic separation of this RNA in polyacrylamide gels of 2.7% (Grootegoed et al., 1977a).

The RNA of the spermatocytes was labelled in tubular fragments which were incubated for 8 h either with (5-^{3}H)uridine of a specific radioactivity of 30 Ci/mmol at a concentration of 20 µCi/ml or with (2-^{14}C)uridine of a specific radioactivity of 60 mCi/mmol at a concentration of 2 µCi/ml.

For autoradiography, spermatocytes were preincubated for 2 h and then incubated for 2 h with (5-^{3}H)uridine of a specific radioactivity of 5 Ci/mmol at a concentration of 5 µCi/ml. The cells were fixed with a mixture of absolute ethanol (3 parts) and glacial acetic acid (1 part) at 0°C, dropped on microscope slides and dried (Kierszenbaum & Tres, 1974). The slides were placed in 5% TCA for 5 min at 0°C and washed in running tapwater for 2 h (Monesi, 1965). The slides were dipped in Ilford Nuclear Emulsion K2 (Ilford Ltd., Ilford, Essex, U.K.) and the film was exposed to the cells for 1 or 8 days at 4°C. After development and fixation of the radioautograms the cells were stained for 30 min with Giemsa et a 1:25 dilution in 0.05 M phosphate buffer, pH 7.2.

RESULTS

Hypophysectomy of immature rats

After hypophysectomy the testicular weight of the immature rats ceased to increase with age and 64 h after hypophysectomy the testicular weight was approx. 65-70% of the testicular weight of intact controls. In periodic-acid-Schiff-haematoxylin stained testicular sections, many pachytene spermatocytes with a dark stained cytoplasm were observed 64 h after hypophysectomy of 26-day old-rats (Fig. 1). When a cell suspension of testes from intact immature rats was separated by velocity sedimentation at unit gravity the pachytene spermatocytes were visible in the sedimentation column as a band sedimenting over 22-26 mm within 80 min at room temperature; 64 h after hypophysectomy this band was hardly visible

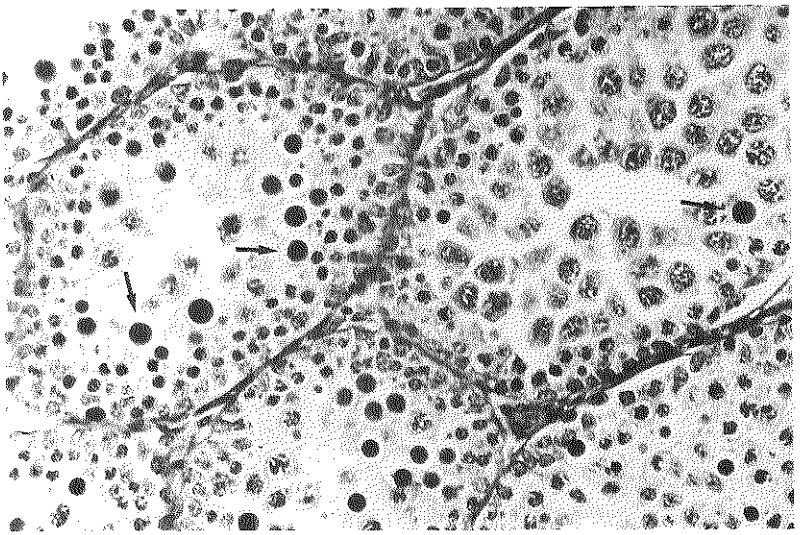

Figure 1. Histology of testicular tissue of a 29-day old rat, 64 h after hypophysectomy, showing the presence of pachytene spermatocytes with a dark-stained cytoplasm (Bouin's fixative, periodic-acid-Schiff-haematoxylin stain; X 200).

against the background. Follitropin and testosterone propionate, injected twice daily in hypophysectomized rats, maintained testicular weight at the level of the intact controls and after separation of cells from the testes of these hormone-treated hypophysectomized rats a visible band of pachytene spermatocytes was present in the sedimentation column.

Uptake and incorporation of ^{3}H-uridine by spermatocytes

The corrected values (correction factors 1.1-1.6) obtained for uptake and incorporation of ^{3}H-uridine by isolated pachytene spermatocytes are presented in Fig. 2. Hypophysectomy caused after 64 h a decrease in uptake of ^{3}H-uridine by spermatocytes and also the incorporation of ^{3}H-uridine into RNA of spermatocytes decreased ($p < 0.01$, two-tailed Student t-test). The ratio of

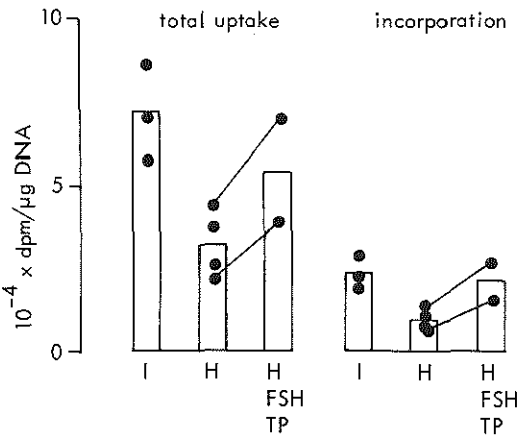

Figure 2. Uptake of ^{3}H-uridine by isolated spermatocytes and incorporation into RNA. Cell preparations (enriched with pachytene spermatocytes and round spermatids respectively) were isolated from 30-35 day-old rats, intact or 64 h after hypophysectomy. In two experiments, the hypophysectomized rats were injected with follitropin and testosterone proprionate as described in Materials and Methods. The isolated cells were incubated for 2 h in the presence of ^{3}H-uridine. The incorporation of ^{3}H-uridine was estimated as dpm in acid-precipitable material. The total uptake of ^{3}H-uridine was estimated as the sum of dpm in acid-soluble and acid-precipitable material. Both values were corrected for the amount of DNA present. The values obtained for spermatocytes, corrected as described in Materials and Methods for the presence of other cell types, are presented. The height of the bars corresponds to the mean result of 2-4 experiments and the dots correspond to the mean result of the single experiments with estimations in triplo. Results of experiments performed simultaneously with rats of the same age are connected via the solid lines.

I - intact rats

H - hypophysectomized rats

H/FSH/TP - hypophysectomized rats treated with follitropin and testosterone proprionate

radioactivity incorporated to the total uptake of radioactivity (0.32, 0.33 and 0.35 for the spermatocytes from intact control rats and 0.26, 0.29, 0.29 and 0.32 for the spermatocytes from hypophysectomized rats) was, however, not markedly decreased after hypophysectomy. Subcutaneous injections of follitropin and testosterone propionate twice daily starting within 4 h after hypophysectomy appeared to largely prevent the decrease in uptake and incorporation of ^{3}H-uridine by the spermatocytes.

Electrophoretic profiles of labelled RNA of spermatocytes

RNA synthesis in spermatocytes present in tubular fragments was studied in tubular fragments from intact and hypophysectomized rats after double-isotope labelling for 8 h. Tubular fragments from intact rats were incubated with ^{14}C-uridine and tubular fragments from rats which were hypophysectomized 48 h before use were labelled with ^{3}H-uridine. After incubation the ^{3}H- and ^{14}C-labelled tubular fragments were combined, pachytene spermatocytes were isolated and RNA was extracted. The electrophoretic profiles of ^{3}H- and ^{14}C-labelled RNA are presented in Fig. 3. These profiles show, that the radioactivity is present in RNA with a heterogeneous electrophoretic mobility ("heterogeneous" RNA) and in peaks of presumptive 45S, 32S, 28S and 18S rRNA. The relative contribution of the different labelled components to the electrophoretic profile was similar for RNA of spermatocytes from intact (^{14}C-profile) and hypophysectomized rats (^{3}H-profile).
The presence of a high peak of labelled 32S rRNA and a low peak of labelled 28S rRNA (Fig. 3) was not observed in round spermatids or Sertoli cells (Grootegoed et al., 1977a). Therefore, most of the labelled RNA in the profiles of Fig. 3 has been formed in the pachytene spermatocytes and not in contaminating spermatids or somatic cell types.

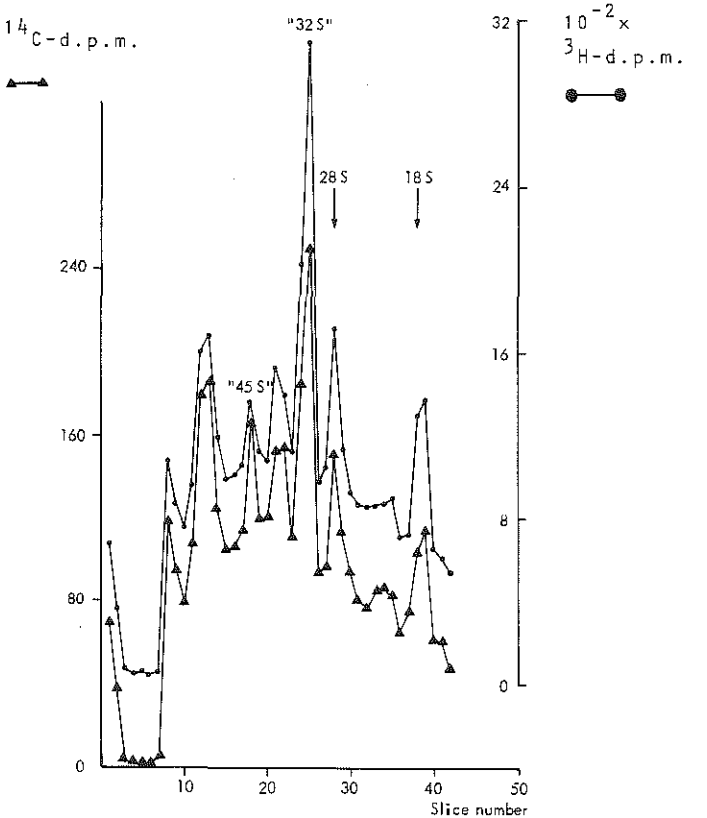

Figure 3. Electrophoretic profiles of ^{3}H- and ^{14}C-uridine incorporation into RNA of spermatocytes present in tubular fragments. Tubular fragments from 26-28 day-old rats (intact or 48 h after hypophysectomy) were incubated for 8 h with ^{14}C-uridine or ^{3}H-uridine. After incubation the tubular fragments from intact and hypophysectomized rats were combined. Spermatocytes were isolated from the combined tubular fragments at 5°C. RNA was extracted from the spermatocytes and separated by electrophoresis in a polyacrylamide gel of 2.7% for 130 min at 3 mA/gel. ^{3}H- and ^{14}C-radioactivity was estimated in gel slices of 1.4 mm. The positions of 28S and 18S rRNA as well as the calculated positions of "32S" and "45S" RNA are indicated.

▲——▲ (^{14}C) in RNA of spermatocytes from intact rats

●——● (^{3}H) in RNA of spermatocytes from rats 48 h after hypophysectomy.

The amount of incorporated ^{3}H-uridine has been estimated after incubation of isolated spermatocytes with ^{3}H-uridine for 2 h (Fig. 2). Almost no rRNA but mainly "heterogeneous" RNA is labelled when isolated spermatocytes are incubated with ^{3}H-uridine for 2 h (Grootegoed et al., 1977a). The electrophoretic mobility of the "heterogeneous" RNA, labelled after incubation of isolated spermatocytes with ^{3}H-uridine for 2 h, was similar 0, 2 or 4 days after hypophysectomy of the rats (results not shown).

Radioautography of isolated spermatocytes, labelled with ^{3}H-uridine

For radioautography the isolated spermatocytes were labelled for 2 h with ^{3}H-uridine. In the radioautograms, stained with Giemsa, the pachytene spermatocytes were identified according to size, structure of the condensed chromatin, the presence of

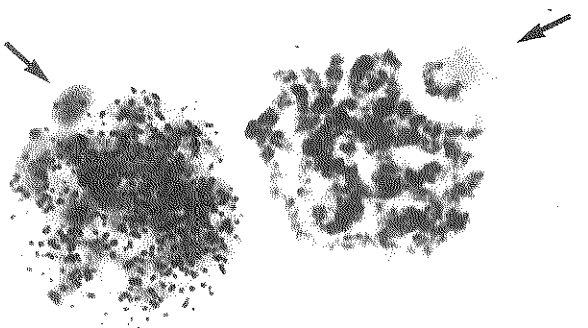

Figure 4. Radioautogram of Giemsa-stained nuclei of isolated pachytene spermatocytes showing spermatocytes with and without grains over the nucleus. The arrows point to the condensed XY chromosomes (exposure time 8 days; X 2000).

one or several nucleoli and the condensed XY chromosomes. Grains were present over the whole body of the nucleus, also the nucleoli showed labelling, but the condensed XY chromosomes were unlabelled (Fig. 4). Unlabelled pachytene spermatocytes were only counted if the structure of the chromatin and the presence of the condensed XY chromosomes allowed identification (Fig. 4). In radioautograms, exposed for 8 days, the number of completely unlabelled pachytene spermatocytes, expressed as percentage of total number of pachytene spermatocytes, was 3 ± 2% (mean ± S.D., n = 5) in preparations of spermatocytes from intact rats and 21% and 25% in two preparations of spermatocytes from rats 64 h after hypophysectomy (600 spermatocyte nuclei counted per cell preparation). The number of grains per spermatocyte nucleus was counted in radioautograms, exposed for 1 day (Fig. 5). After hypophysectomy the radioautograms showed the presence of a relatively large number of (almost) unlabelled pachytene spermatocytes, and the number of pachytene spermatocytes with a high activity (41 and more grains per nucleus) was relatively low (Fig. 5).

DISCUSSION

The present study was undertaken to investigate the possible effect of hormones on RNA synthesis in pachytene spermatocytes. We have examined the incorporation of ^{3}H-uridine into RNA of cell preparations enriched in pachytene spermatocytes isolated from intact and hypophysectomized immature rats.

The uptake of ^{3}H-uridine by preparations of spermatocytes as well as the incorporation of ^{3}H-uridine into RNA were decreased in cells isolated from hypophysectomized rats as compared to cells from intact rats. Administration of follitropin and testosterone propionate to hypophysectomized rats largely prevented this decrease in uptake and incorporation, which may indicate that the absence of

either one or both of these hormones may have caused the effects observed after hypophysectomy. The ratio of incorporation to uptake was not markedly decreased after hypophysectomy, which reflects that the decreased incorporation of ^{3}H-uridine may depend on changes in the uptake. We have no information about the pool-size of RNA precursors but the close correlation between incorporation and uptake of ^{3}H-uridine may suggest that the decreased incorpora-

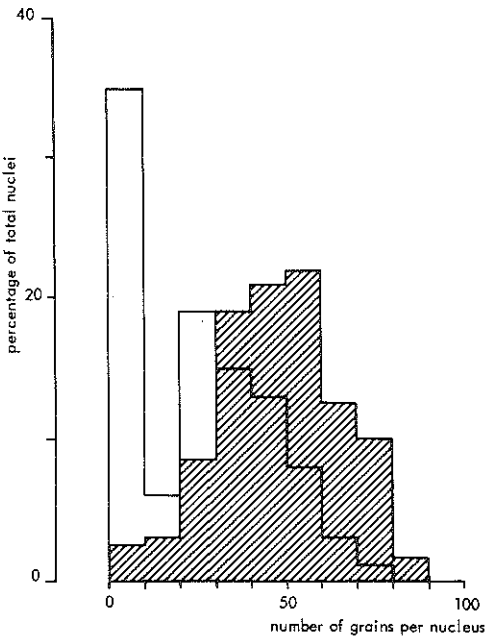

Figure 5. Number of grains per nucleus in radioautograms of isolated spermatocytes, labelled with ^{3}H-uridine. Spermatocytes were isolated from 29-day old rats (intact or 64 h after hypophysectomy). The spermatocytes were incubated for 2 h in the presence of ^{3}H-uridine. The number of grains per nucleus of at least 200 pachytene spermatocytes was counted in radioautograms (8 per cell preparation, exposed for 1 day). The number of nuclei with a corresponding number of grains is presented in the histograms as percentage of total nuclei.
hatched histogram - spermatocytes from intact rats
open histogram - spermatocytes from rats, 64 h after hypophysectomy

tion of ^{3}H-uridine does not represent a change in the relative rate of uridine incorporation.

Electrophoretic profiles of labelled RNA of pachytene spermatocytes from intact and hypophysectomized rats appeared to be similar (Fig. 3). Hence, we have concluded that testes of hypophysectomized rats contain a considerable number of spermatocytes which are intact with respect to the synthesis and turnover of "heterogeneous" RNA as well as the synthesis and processing of rRNA precursors.

Radioautography was used to detect whether or not an effect of hypophysectomy on the incorporation of ^{3}H-uridine was reflected in all isolated spermatocytes. We observed variations in the incorporation of ^{3}H-uridine in individual spermatocytes (Fig. 5). Such variations may be a consequence of the presence of pachytene spermatocytes from different stages of the cycle of the seminiferous epithelium (Leblond & Clermont, 1952), which are known to incorporate ^{3}H-uridine at different rates (Söderström & Parvinen, 1976). Whether or not the isolated spermatocytes represent the population of pachytene spermatocytes *in situ* has, however, not been experimentally verified. After hypophysectomy, it was found that the number of unlabelled spermatocytes in the preparations of isolated cells was increased. On basis of the quantitative evaluation of the radioautographic experiments it may be suggested that one effect of hypophysectomy on ^{3}H-uridine incorporation in pachytene spermatocytes is reflected in a reduction of the number of highly active spermatocytes (41 or more grains per nucleus) together with an increase in the number of unlabelled spermatocytes. The origin of the inactive spermatocytes is not clear; they could have descended either from spermatocytes with varying activity or mainly from the most active spermatocytes. The latter possibility seems to be supported by the observation of Russell & Clermont (1977)

that the pachytene spermatocytes degenerate in the adult rat testis 5.5 days after hypophysectomy at stages VII-VIII of the cycle of the seminiferous epithelium and from radioautographic experiments it can be concluded that at these stages rat pachytene spermatocytes are most active with respect to ^{3}H-uridine incorporation (Söderström & Parvinen, 1976). After hypophysectomy, the increased number of unlabelled spermatocytes in the radioautograms partly explains that the uptake and incorporation of ^{3}H-uridine by the spermatocyte preparations is reduced in the absence of a marked decrease of the relative rate of uridine incorporation. We cannot be certain, however, that the uptake of ^{3}H-uridine was somewhat decreased in the spermatocytes which still appear to synthesize RNA after hypophysectomy.

Several reports have been published on changes in RNA synthesis, RNA content and RNA polymerase activity in the testis, measured in vivo or in vitro after in vivo stimulation with follitropin (Means, 1971; Abney, Skipper & Williams, 1974; Reddy & Villee, 1975) or testosterone propionate (Chiu, Thomson & Hnilica, 1976). In these studies no attempts were made, however, to correlate the observed effects with the presence of specific cell types and pachytene spermatocytes may not have been involved in the observed biochemical changes. Parvinen & Söderström (1976) have reported an increased incorporation of ^{3}H-uridine within 2½ h at all stages of the cycle of the seminiferous epithelium when tubular frameents, isolated from adult rats 3 days after hypophysectomy, were incubated in the presence of follitropin and testosterone. These authors concluded that the rate of RNA synthesis in the tubular fragments was increased. Primary spermatocytes significantly contribute to the incorporation of ^{3}H-uridine in isolated tubular fragments (Söderström & Parvinen, 1976) and this could reflect that the rate of RNA synthesis in these germinal cells is increased. In the inter-

pretation of these results it should be considered, however, that the observed hormone-stimulated incorporation of ^{3}H-uridine is possibly correlated with changes in the uptake of ^{3}H-uridine by the tubular fragments and does not necessarily reflect a change in the rate of RNA synthesis.

In cross-sections of tubules stained with periodic-acid-Schiff-haematoxylin, we have observed within 2-3 days after hypophysectomy an increased number of pachytene spermatocytes with a dark stained cytoplasm. Such cells with a dark stained cytoplasm, which may represent the unlabelled spermatocytes observed in the radioautograms, were not evenly distributed over all tubular cross-sections (Fig. 1). Degeneration of spermatocytes in the testes of immature hypophysectomized rats may therefore occur at defined stages of the seminiferous epithelium as was shown by Russell & Clermont (1976) for adult hypophysectomized rats. After hypophysectomy, the selective degradation of pachytene spermatocytes at a defined stage is increased (Russell & Clermont, 1976) but there is no regression of all spermatocytes at a particular stage, even several weeks after hypophysectomy (Clermont & Morgentaler, 1955).

On basis of the present observations on pachytene spermatocytes of immature rats it appears that hypophysectomy has an effect on the number of pachytene spermatocytes which is maintained and can develop, although no effects on RNA synthesis in pachytene spermatocytes were observed. The number of germinal cells which develop may be dependent on the activity of the Sertoli cells which, as outlined in the Introduction, are target cells for follitropin and testosterone. However, there is as yet no convincing evidence that RNA synthesis in the germinal cells is dependent on the action of follitropin and testosterone on Sertoli cells.

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REFERENCES

Abney, T.O., Skipper, J.K. & Williams, W.L. (1974). Gonadotropin stimulation of rat testicular protein synthesis. Polysome isolation and activity in a cell-free system. Biochemistry 13, 3956-3962.

Chiu, J.F., Thomson, J. & Hnilica, L.S. (1976). Testicular chromatin activation in hypophysectomized rats. Biochimica et Biophysica Acta 435, 1-12.

Clermont, Y. & Harvey, S.C. (1967). Effects of hormones on spermatogenesis in the rat. Ciba Foundation Colloquia on Endocrinology 16, 173-189.

Clermont, Y. and Morgentaler, H. (1955). Quantitative study of spermatogenesis in the hypophysectomized rat. Endocrinology 57, 369-382.

Courot, M. (1970). Effect of gonadotropins on the seminiferous tubules of the immature testis. In: The human Testis, pp. 355-364. Eds E. Rosemberg & C.A. Paulsen. Plenum Press, New York & London.

Grootegoed, J.A., Grollé-Hey, A.H., Rommerts, F.F.G. & van der Molen, H.J. (1977a). Ribonucleic acid synthesis in vitro in primary spermatocytes isolated from rat testis. The Biochemical Journal, 168, 23-31.

Grootegoed, J.A., Peters, M.J., Mulder, E., Rommerts, F.F.G. & van der Molen, H.J. (1977b). Absence of a nuclear androgen receptor in isolated germinal cells of rat testis. Molecular and Cellular Endocrinology, 9, 159-167.

Kierszenbaum, A.L. & Tres, L.L. (1974). Nucleolar and perichromosomal RNA synthesis during meiotic prophase in the mouse testis. The Journal of Cell Biology 60, 39-53.

Leblond, C.P. & Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithilium in the rat. Annals of the New York Academy of Sciences 55, 548-573.

Louis, B.G. & Fritz, I.B. (1977). Stimulation by androgens of the production of androgen binding protein by cultured Sertoli cells. Molecular and Cellular Endocrinology 7, 9-16.

Means, A.R. (1971). Concerning the mechanism of FSH action: Rapid stimulation of testicular synthesis of nuclear RNA. Endocrinology 89, 981-989.

Means, A.R. (1975). Biochemical effects of follicle-stimulating hormone on the testis. In: Handbook of Physiology, section 7, volume V: Male reproductive system, pp. 203-223. Volume eds D.W. Hamilton & R.O. Greep. American Physiological Society, Washington, D.C.

Monesi, V. (1965). Synthetic activities during spermatogenesis in the mouse. Experimental Cell Research 39, 197-224.

Parvinen, M. & Söderström, K.-O. (1976). Effects of FSH and testosterone on the RNA synthesis in different stages of rat spermatogenesis. Journal of Steroid Biochemistry 7, 1021-1023.

Platz, R.D., Grimes, S.R., Meistrich, M.L. & Hnilica, L.S. (1975). Changes in nuclear proteins of rat testis cells separated by velocity sedimentation. The Journal of Biological Chemistry 250, 5791-5800.

Reddy, P.R.K. & Villee, C.A. (1975). Messenger RNA synthesis in the testis of immature rats: effect of gonadotropins and cyclic AMP. Biochemical and Biophysical Research Communications 63, 1063-1069.

Russell, L.D. & Clermont, Y. (1977). Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. Anatomical Record 187, 347-366.

Söderstrom, K.-O. & Parvinen (1976). RNA synthesis in different stages of rat seminiferous epithelial cycle. Molecular and Cellular Endocrinology 5, 181-199.