ROLE OF CARBOHYDRATE METABOLISM IN HORMONAL REGULATION OF SPERMATOGENESIS

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II. N.H.P.M. Jutte, J.A. Grootegoed, F.F.G. Rommerts & H.J. van der Molen: "Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids"; J. Reprod. Fert. 62 (1981) 399-405.

Appendix Papers (continued)

III. N.H.P.M. Jutte, R. Jansen, J.A. Grootegoed, F.F.G. Rommerts, O.P.F. Clausen & H.J. van der Molen: "Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells"; J. Reprod. Fert. 65 (1982) 431-438.

IV. N.H.P.M. Jutte, R. Jansen, J.A. Grootegoed, F.F.G. Rommerts & H.J. van der Molen: "FSH stimulation of the secretion of pyruvate and lactate by Sertoli cells may be involved in hormonal regulation of spermatogenesis"; (submitted to J. Reprod. Fert.).

Chapter 1

INTRODUCTION

1.1. Some aspects of spermatogenesis

In mammals the testis, the site of male germ cell development, can be divided morphologically in two cellular compartments, viz. the seminiferous tubules and the interstitial tissue between the tubules. The seminiferous tubules contain developing germ cells and Sertoli cells and are surrounded by a boundary layer of myoid cells. The interstitial tissue contains Leydig cells, blood and lymph vessels, nerves and fibroblasts. In intact animals transfer between the two compartments is restricted (Setchell & Waites, 1975). A barrier to substances of widely varying molecular size is formed, because the Sertoli cells which line the seminiferous tubules are closely connected by elaborate tight junctions between the basal parts of the cells (blood-testis barrier). In some species a second, less effective barrier is formed by myoid cells which surround the seminiferous tubules (Fawcett, 1975) (Figure 1.1).

Development of germ cells starts with the spermatogonial stem cells which are located at the basis of the Sertoli cells in the seminiferous tubules but outside the blood-testis barrier (basal compartment). After several mitotic divisions of the spermatogonia, preleptotene spermatocytes develop which synthesize DNA, so that they finally contain twice the amount of DNA present in non-dividing somatic cells. In the prophase following the preleptotene stage, rearrangement of the chromosomal material takes place as a preparation for the first meiotic division. The meiotic prophase is subdivided in the leptotene, zygotene, pachytene and diplotene. During the leptotene and zygotene tight junctions are formed between the Sertoli cells at the basal side of the germ cells and subsequently the tight junctions at the luminal side are dissolved (Russell, 1980). In this way the germ cells are transported through the blood-testis barrier to the lumen of the seminiferous tubules (adluminal compartment) without disruption of the blood-testis barrier. The prophase is followed by the first meiotic division, and the generated secondary spermatocytes go quickly through the second meiotic division, without synthesis of DNA. The resulting

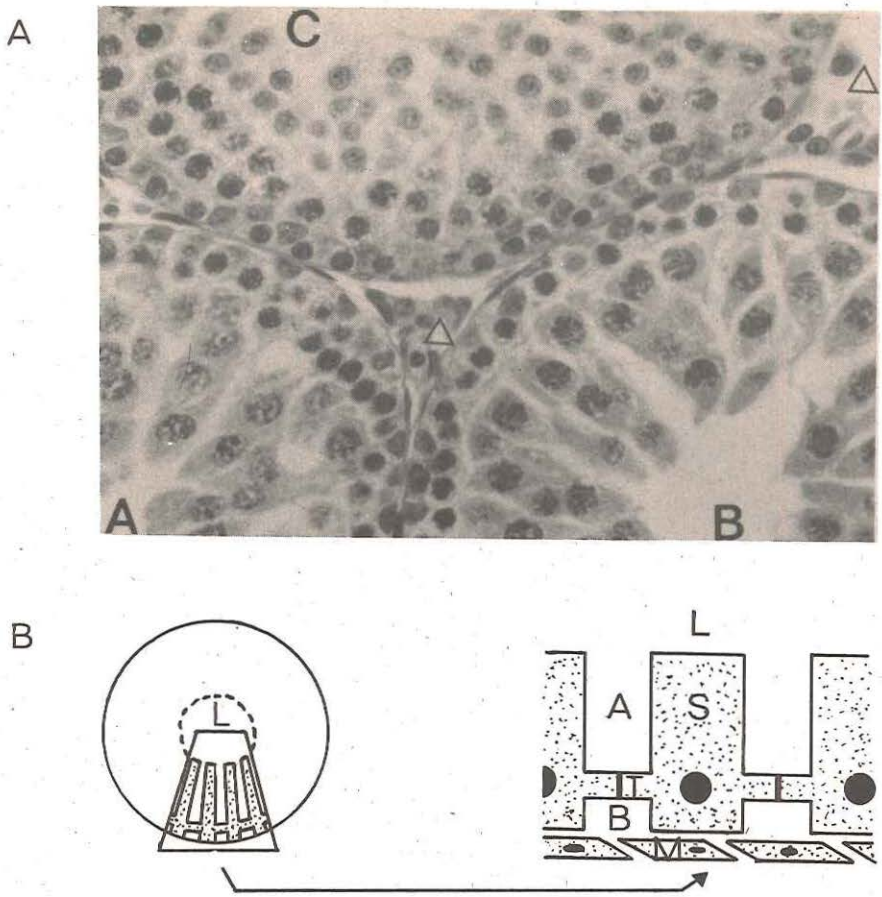


Figure 1.1. a) Transverse section of a small fragment of testicular tissue from a 32-day-old (immature) rat (x1400).
Spermatogenesis is still incomplete at this age. In the fragments of seminiferous tubules shown, the most advanced germ cells, which are located at the luminal side of the tubules, are late pachytene spermatocytes (tubules A and B) or round spermatids (tubule C). Between the tubules the interstitial tissue can be observed (Δ) which contains Leydig cells, blood and lymph vessels, and fibroblasts.

b) Scheme of the position of the cells in the germinal epithelium and the adjacent cell layer.
Sertoli cells (S) and myoid cells (M) line the basal compartment (B), the location of spermatogonia and preleptotene spermatocytes. During germ cell development leptotene and zygotene spermatocytes pass through the tight junctions (T) between the Sertoli cells, and the more mature germ cells are located in the adluminal compartment (A), surrounded by Sertoli cells. Finally, the spermatozoa are transported from the testis via the lumen (L) of the seminiferous tubules.

haploid spermatids, which contain half the amount of DNA present in non-dividing somatic cells, differentiate from round cells to spermatozoa involving nuclear and cytoplasmic elongation and reorganization. The close contact between the developing germ cells and the Sertoli cells is then lost and the spermatozoa are released into the lumen of the tubule and are transported from the testis. During spermatogenesis the germ cells remain interconnected by cytoplasmatic bridges, which remain after the mitotic divisions of the spermatogonia and which are broken only during spermatid elongation.

The entry of spermatogonia into meiosis occurs at fixed intervals, lasting approximately 13 days in the rat. Total development from spermatogonium to spermatozoon also has a fixed length which is approximately 50 days for rats. Therefore in a given tubule section different generations of germ cells are present which occur in cellular associations of a fixed composition. For the rat 14 associations have been distinguished, based on the morphology of the differentiating spermatids (Leblond & Clermont, 1952).

Sertoli cells enclose almost all developing germ cells within the blood-testis barrier. In the immature animal, differentiation beyond the spermatocyte stage is temporally correlated with the development of Sertoli cell-tight junctions (Fawcett, 1974). This may indicate that the barrier is important for spermatogenesis. It is thought that Sertoli cells contribute to the composition of the tubular fluid by active secretion (Fritz, 1978; Waites & Gladwell, 1982). Extensions of Sertoli cell cytoplasm surround all germ cells and cytoplasmic and membrane specializations were shown on the boundary of Sertoli cells and the adjacent germ cells (review: Russell, 1980). Based mainly on such morphological evidence it is generally thought that the presence of Sertoli cells is obligatory for spermatogenesis. As possible explanations for the action of Sertoli cells upon germ cells it has been suggested, that Sertoli cells may render mechanical support, nutrition or control of differentiation.

Spermatogenesis is dependent on the pituitary hormones follitropin (FSH) and lutropin (LH). LH acts upon spermatogenesis via its stimulation of testosterone synthesis by Leydig cells. In adult hypophysectomized rats, the absence of FSH and testosterone results in an increased degeneration of advanced germ cells, viz. mid-pachytene spermatocytes, round spermatids step 7 and elongating spermatids step 19 (Russell &

Clermont, 1977). Ample research (mainly on rats) has been performed to learn which part of germ cell development is influenced by the different hormones. The literature on this subject has been reviewed extensively by Steinberger (1971) and Fritz (1978). In summary, it is thought now that the presence of FSH is mainly required during initiation of spermatogenesis in immature rats and during restoration of spermatogenesis in adult animals. Testosterone is thought to be most important for maintenance of spermatogenesis in mature animals. However, in addition testosterone possibly facilitates initiation of spermatogenesis in immature rats when FSH is present, and FSH may cooperate with testosterone to maintain spermatogenesis in mature rats. With regard to the hormonal regulation of spermatogenesis it is important to distinguish qualitative regulation, i.e. the influence of hormones on the spermatogenic process per se, and quantitative regulation, i.e. the effect on the number of germ cells which develop.

Secretion of androgen binding protein by Sertoli cells is correlated with the state of spermatogenesis under different hormonal conditions, suggesting that spermatogenesis requires functioning Sertoli cells (review: Purvis & Hansson, 1981). FSH exerts many effects on Sertoli cell activities and these effects have been reviewed recently (Means et al., 1980; Davies, 1981; Purvis & Hansson, 1981; Ritzén et al., 1981; Waites & Gladwell, 1982). No direct effects of FSH on germ cells have been reported, although FSH binding to spermatogonia was suggested (Orth & Christensen, 1978). Androgen receptors were shown to be present in Sertoli cells (Mulder et al., 1976), but were absent in spermatocytes and spermatids (Grootegoed et al., 1977b). Several observations, which support the absence of a direct androgen effect on germ cells, were recently reviewed (Fritz, 1978). Therefore, at the moment it is generally assumed that the effects of FSH and testosterone on spermatogenesis are mediated by Sertoli cells (Grootegoed et al., 1977b; Fritz, 1978; Ritzén et al., 1981). Still, the mechanism by which Sertoli cells act upon germ cells remains to be elucidated.

1.2. Aim and scope of the present thesis

The assumption that the effect of hormones on spermatogenesis is mediated by Sertoli cells is based on rather indirect evidence because it remains unclear in which way Sertoli cells may influence germ cells and which biochemical events in germ cells may be influenced by Sertoli cells. To gain more insight into these questions, investigations with isolated Sertoli cell and germ cell preparations were performed (Chapter 2). We have investigated in particular pachytene spermatocytes and round spermatids, two germ cell stages which are sensitive to the absence of FSH and testosterone and to other conditions that disturb spermatogenesis, like hypoglycemia and heat (Clermont & Morgentaler, 1955; Mancini et al., 1960; review: Setchell, 1978). Moreover, these two cell types can be isolated from rat testes.

Specific changes in cells will often be expressed in a change of proteins synthesized. The development of advanced two-dimensional electrophoretic techniques (O'Farrell, 1975) has made it possible to visualize small changes in protein synthesis. We investigated whether protein synthesis in germ cells, incubated in the absence of Sertoli cells, changed, to gain insight into the dependence of germ cells on Sertoli cells (Chapter 3). The rate of protein synthesis in adult testicular tissue was previously shown to be extremely dependent on glucose supply when compared to other tissues (Davis, 1969). It was concluded from experiments with total testicular tissue, that pachytene spermatocytes and round spermatids required glucose (Means & Hall, 1968; Davis, 1969). Preliminary investigations on isolated germ cells, however, indicated that synthetic processes in pachytene spermatocytes and round spermatids were hardly stimulated by glucose, but were markedly stimulated by catabolites of glucose (Chapter 4.1). It seemed that in total testicular tissue the effect of glucose on germ cells could be mediated by the glucose catabolism of Sertoli cells (Figure 1.2). This phenomenon was the first known reaction of germ cells on a controllable and possibly physiologic stimulus that might be regulated by Sertoli cells. In this regard we have studied some aspects of carbohydrate metabolism in isolated germ cells and Sertoli cells (Chapter 4.1, 4.2), and we have investigated a possible carbohydrate-mediated relationship between Sertoli cells and germ cells (Chapter 4.3). Finally, a possible role of carbohydrate metabolism in the effect of hormones on germ cells was investigated (Chapter 4.2, 4.3).

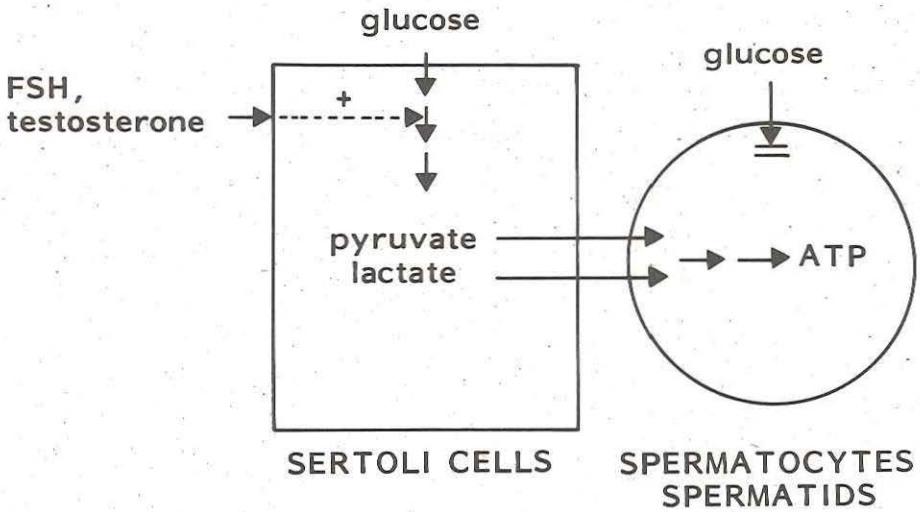


Figure 1.2. Hypothetical model for the effect of glucose on synthetic processes in germ cells.
Glucose was found to stimulate protein synthesis of germ cells present in testicular tissue. However, protein synthesis in isolated germ cells was hardly increased by glucose and stimulation by pyruvate and lactate was far more effective. Possibly, Sertoli cells exert a positive effect on germ cells via a (hormone-dependent) supply of pyruvate and lactate.

Chapter 2

MATERIALS AND METHODS

A summary of some general aspects of the methods is presented in this chapter. Details of the materials and methods used in the present experiments have been described in detail in the appendix papers.

Animals

Pachytene spermatocytes and round spermatids were isolated from testes of immature rats (30-35 days old). At this age spermatogenesis has progressed up to and including development of round spermatids and no elongated spermatids or spermatozoa have been formed, which during purification could sediment with pachytene spermatocytes and round spermatids.

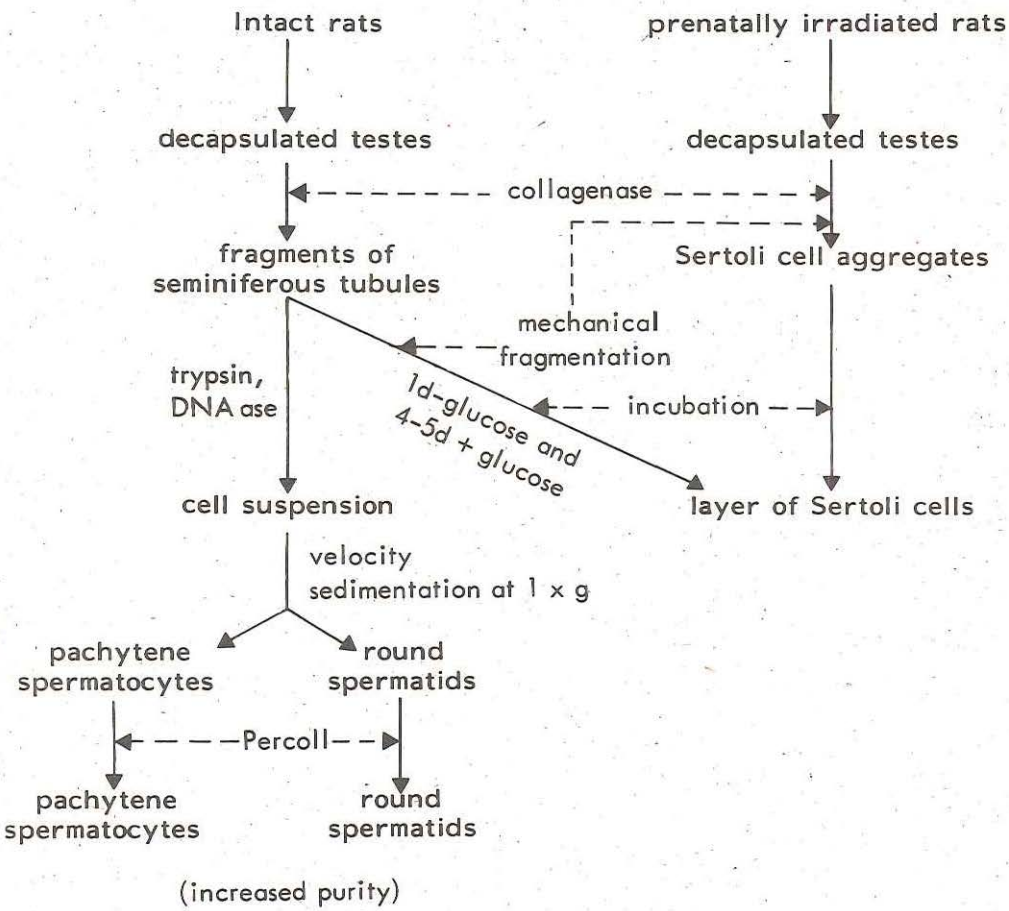
Sertoli cells were isolated from testes of rats of different ages, which were depleted of germ cells after irradiation in utero on day 19 of gestation (Beaumont, 1960). At this age the primordial germ cells are very sensitive to irradiation and degenerate when they attempt to divide. Therefore, this treatment results in a sterile testis. It has been shown that in these Sertoli cell-enriched testes from rats below 20 days of age, FSH binding, adenylate cyclase and protein kinase activation, and ABP production take place like in testes of normal rats. However, formation of the blood testis barrier appears to be delayed for approximately 10 days (reviews: Means et al., 1976; Means et al., 1978). It was observed in our experiments that after a few months some gonocytes started to divide and spermatogenesis could take place in several tubules. This indicates that after irradiation Sertoli cells remain capable to support spermatogenesis.

Seminiferous tubules were generally obtained from testes of 30-35 day old rats. In some experiments, testes of 25 day old rats were used. At this age not many round spermatids have formed in the testes and spermatogenesis has progressed up to and including pachytene spermatocytes.

For one experiment rats of 25 days of age were hypophysectomized via the auditory canal.

The isolation of germ cells and tubular fragments

An outline of the isolation procedure is given in the following scheme.



The distribution of cells over the fractions obtained after sedimentation of the cell suspension at 1 x g is shown in Figure 2.1. Details of the procedures for the isolation of germ cells and Sertoli cells have been presented in Appendix Paper III. The additional purification of the isolated germ cells by centrifugation in a Percoll gradient has been used for the experiments described in Chapter 3 and Appendix Paper I. The procedure has been described in Appendix Paper I.

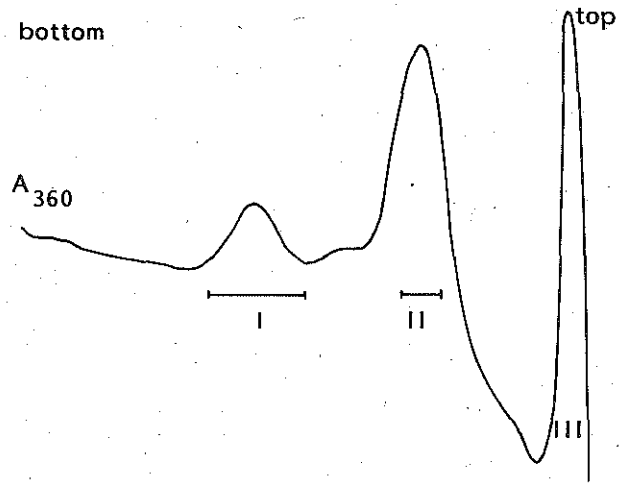


Figure 2.1. Distribution of cells eluted from a sedimentation column used for enrichment of germ cells.
A germ cell suspension was obtained from testicular tissue of 32 day old rats as described in Appendix Paper III. After sedimentation of the cells in a non-linear albumin gradient (1-3.2%) (Grootegoed et al., 1977a) for 75 min at room temperature, the column was eluted from the bottom at a rate of 15 ml per min. The eluate was lead through a cuvette which was placed in a spectrophotometer and the transmission, measured at 360 nm, was monitored continuously with a recorder. Simultaneously, fractions of 10 ml were collected and pooled as indicated in the figure. The peaks contained predominantly pachytene spermatocytes (I), round spermatids (II) or somatic cells and cellular debris (III) (see Appendix Paper IV).

Media used for isolation and incubation of cells

The isolation and incubation medium were respectively essentially Hank's Balanced Salt Solution (Hanks & Wallace, 1949) and Eagle's Minimal Essential Medium (Eagle, 1959). Both media were modified with an increased amount of KCl (56.9 mM) and the osmolarity was adjusted by lowering the NaCl concentration (Grootegoed et al., 1977a). The isolation medium was supplemented with 6 mM sodium DL-lactate and 2 mM sodium pyruvate. The incubation medium contained 3.3 mM glucose, and no pyruvate and lactate, unless described otherwise at the individual experiments. For estimation of oxygen consumption, cells have been incubated in phosphate-buffered saline (Dulbecco & Vogt, 1954), supplemented with vitamins and amino acids as present in the incubation medium.

Incubation conditions

Incubations were performed at 32°C under a humidified atmosphere of 5 % CO_2 in air. The measurements of germ cell activities were generally started within 1 h after completion of the isolation procedure and the incubation period was 2 h, unless described otherwise. The activities of Sertoli cells were generally estimated during 24-48 h after completion of the isolation procedure. After the first period of 24 h the medium was renewed.

Experiments were performed by incubation of isolated cell types or combinations of different cell types according to the following scheme.

isolated cells	combination of cells
germ cells	seminiferous tubules
Sertoli cells	isolated Sertoli cells + isolated germ cells (recombination)
germ cells in spent medium from Sertoli cells (conditioned medium)	

Analysis of cellular activities

Qualitative analysis of cellular protein synthesis was performed, after incubation of the cells for 2 h in the presence of (^{35}S)methionine, by means of one- and twodimensional electrophoresis as described in Appendix Paper II.

Oxygen consumption of intact cells was measured in a Warburg apparatus (Umbreit et al., 1964).

Quantitative analysis of cellular RNA and/or protein synthesis was performed by estimating the amount of radioactively labelled precursors, incorporated during 2 h in acid-precipitable material, as described in Appendix Papers II and III.

Glucose, pyruvate and lactate were estimated in media from germ cells incubated from 0-24 h and in media from Sertoli cells incubated from 24-48 h after isolation, unless described otherwise. Glucose was estimated using enzymic conversion with hexokinase and glucose-6-phosphate dehydrogenase (Schmidt, 1961). Pyruvate and lactate were estimated enzymically, using lactate dehydrogenase (Czok & Lamprecht, 1970; Hohorst, 1970).

Analysis of the composition of cell preparations

The cellular composition of the preparations of isolated germ cells was monitored after fixing the cells in Bouin's fixative on microscopic slides and staining with hematoxylin.

The cellular composition of fragments of seminiferous tubules was estimated by DNA flow cytometry immediately after isolation or following incubation. After dispersion of the cells by enzymic treatment, which also destroyed dead cells, the DNA of the intact nuclei can be stained with ethidiumbromide (Appendix Paper III). Nuclei of cells of the germinal epithelium contain different amounts of DNA (viz. primary spermatocytes: 4C, Sertoli cells, secondary spermatocytes and spermatogonia: 2C, and spermatids: 1C; 1C is defined as the amount of DNA present in a haploid gamete), and showed different fluorescence intensities after staining with ethidiumbromide. During processing of the suspension through a cytofluorometer, the counting of the fluorescent nuclei was interrupted after the fluorescence in the highest peak had reached a maximal level. Therefore, the distribution of fluorescence over the different peaks was relative, and the number of germ cells and Sertoli cells can be expressed as percentage of the total number of cells (Figure 2.2). To facilitate comparison between different cell suspensions, the number of germinal cells was expressed relative to the number of Sertoli cells in some experiments.

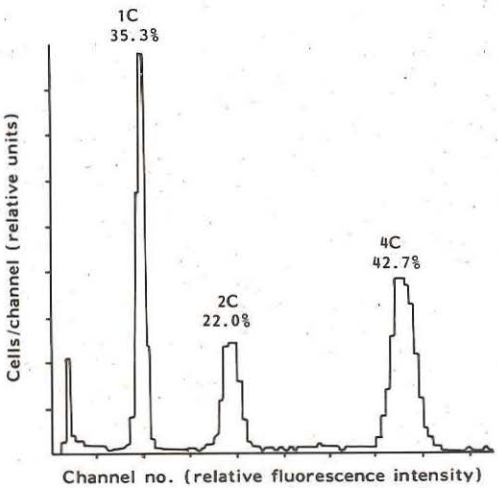


Figure 2.1. DNA distribution pattern of dispersed cells obtained from fragments of seminiferous tubules of 33-day-old rats after incubation for 1 day. Nuclei of primary spermatocytes (4C), and somatic cells (mostly Sertoli cells), secondary spermatocytes and spermatogonia (2C) and spermatids (1C) showed fluorescence intensities corresponding to their DNA content (see above). The amount of each cell type was calculated as percentage of the total number of cells.

Chapter 3

PROTEIN SYNTHESIS IN GERM CELLS

3.1. Differences in protein synthesis in different germ cell types

Description of the morphology of germ cell development was followed by investigation of several biochemical parameters of this complicated series of events (reviews: Bellvé, 1979; Erickson et al., 1981). With autoradiography it was shown that protein synthesis occurs at all germ cell stages (rat: Davis, 1969; mouse: Monesi, 1971; ram: Loir, 1972).

Nuclear basic proteins are thought to play a role in regulation of nuclear events and were studied extensively, which has resulted in the discovery of testis-specific histones in germ cells in addition to the regular somatic histones. During spermatid elongation the histones are replaced by testis-specific non-histone basic proteins, which are subsequently replaced by arginine- and cysteine-rich protamine. The role of the testis-specific histones in germ cell development is not yet clear, despite recent studies on histones concerning site of synthesis (Brock et al., 1980; Meistrich et al., 1981), isolation, characterization and quantification (Chiu & Irvin, 1980; Kumaroo & Irvin, 1980; Seyedin & Kistler, 1980; Trostle-Weige et al., 1982) and organ specificity (Seyedin & Kistler, 1979). The non-histone basic proteins are possibly involved in stabilization and condensation of the nuclear material (review: Bellvé, 1979; Rodman et al., 1979). Nuclear non-histone acidic proteins have not been intensively investigated (Bellvé, 1979).

The abundance of some specific proteins in spermatozoa initiated studies concerning their presence and site of synthesis during spermatogenesis. These studies were performed mainly by correlating the appearance of proteins in total testicular tissue with the appearance of particular germ cell stages or by histochemical detection. A special group of proteins are the testis-specific isozymes, which emerge in germ cells during spermatogenesis (reviews: Goldberg, 1977; Bellvé, 1979; Blanco, 1980; Erickson et al., 1981). The occurrence of specific protein synthesis in cells before or after meiosis was often used to investigate whether postmeiotic gene expression occurred. However, definite proof for haploid

gene expression is still lacking (Erickson et al., 1981).

Biochemical analysis of germ cells has progressed through the increasing use of cell separation techniques. With respect to proteins, recent studies, performed on different germ cell preparations enriched with one stage of development, involved characterization of total cellular proteins (Boitani et al., 1980; Geremia et al., 1981; Kramer & Erickson, 1982), mitochondrial proteins (Hecht & Bradley, 1981), membrane proteins (Millette & Moulding, 1981a,b), glycoproteins (Grootegoed et al., 1982c) or specific proteins, such as specific membrane antigens (Tung & Fritz, 1978; Millette & Bellvé, 1980; O'Rand & Romrell, 1981; Gaunt, 1982; Romrell et al., 1982), adenylate cyclase (Adamo et al., 1980; Gordeladze et al., 1981), protein kinase (Conti et al., 1979), DNA polymerase (Grippo et al., 1978; Hecht et al., 1979), protein carboxymethylase (Gagnon et al., 1979), phosphoglycerate kinase (Kramer & Erickson, 1981) and glycosyltransferases (Letts et al., 1978).

In our studies we were interested in a biochemical "fingerprint" of different germ cell stages as a potential parameter for the investigation of the action of Sertoli cells upon germ cells. With two-dimensional electrophoresis several hundreds of proteins could be separated.
With this method we investigated protein synthesis in cell preparations highly enriched with pachytene spermatocytes and round spermatids and the results, shown in Appendix Paper I, demonstrated clear differences between the two cell types. Some proteins were synthesized predominantly in pachytene spermatocytes, some predominantly in round spermatids and a large group of proteins was synthesized at the same rate in spermatocytes and spermatids. The synthesis of total cellular proteins in these germ cell stages has also been analyzed by others (Boitani et al., 1980; Kramer & Erickson, 1981). All studies demonstrated synthesis of stage-specific proteins, although the number varied in the different cells, possibly as a result of differences in experimental conditions. Because we obtained with two-dimensional electrophoresis a specific pattern of proteins newly synthesized by pachytene spermatocytes, this technique was used to investigate the protein synthesis in spermatocytes under different conditions.

3.2. Maintenance of qualitative protein synthesis in isolated pachytene spermatocytes

The presence of Sertoli cells appears obligatory for spermatogenesis. It is largely unknown, however, which biochemical activities of germ cells are primarily dependent on activities of Sertoli cells. We have investigated whether changes were induced in protein synthesis of pachytene spermatocytes during incubation of 24 hours in the absence of Sertoli cells, in medium containing pyruvate (2 mM) and DL-lactate (6 mM). As shown in Appendix Paper I, the results demonstrated that the pattern of proteins, newly synthesized by isolated pachytene spermatocytes immediately after isolation, and analyzed with two-dimensional electrophoresis, was maintained during 24 h. Moreover, the amount of amino acids incorporated into acid-precipitable material was comparable for spermatocytes incubated from 0-2 h or from 24-26 h in the presence of pyruvate and lactate. In contrast, the pattern of proteins synthesized in Sertoli cells was shown to change during one day of incubation.

Previously it was reported, that isolation of mouse pachytene spermatocytes and spermatids from seminiferous tubules did not result in an immediate change of the protein synthesis pattern (Boitani et al., 1980). Moreover, it was shown that qualitative RNA synthesis by isolated spermatocytes and glycoprotein fucosylation by isolated spermatids were maintained after respectively 12 h and 20 h of incubation in chemically defined medium (Grootegoed et al., 1977a; Grootegoed et al., 1982a). These observations demonstrate that pachytene spermatocytes and round spermatids incubated in a chemically defined medium, can maintain a specific pattern of synthetic activities in the absence of Sertoli cells. Many activities of Sertoli cells *in vitro* are stimulated by FSH and therefore it seems likely that activities of Sertoli cells *in vivo* are decreased after hypophysectomy. In rats, no changes were observed in qualitative RNA synthesis of pachytene spermatocytes at 64 h after hypophysectomy (Grootegoed et al., 1979). Therefore, spermatocytes appear to maintain their qualitative synthetic activities *in vitro* in the absence of Sertoli cells, and also *in vivo* in the presence of Sertoli cells with a reduced activity.

Chapter 4

CARBOHYDRATE METABOLISM OF GERM CELLS AND SERTOLI CELLS

In intact animals, the development, composition and normal function of the testis are dependent on an adequate supply of several nutrients (review: Leathem, 1975). It is difficult to establish, however, whether the effects of nutrients on the testis are exerted directly on Leydig cells and/or germinal epithelium or occur via an intermediate action of nutrients on the pituitary (Leathem, 1975; Setchell, 1978). This problem can be partly circumvented by investigation of testicular tissue in vitro.

The first in vitro studies on energy metabolism of rat testis already showed that both lipids and carbohydrates were oxidized with the latter predominating. These and following investigations on carbohydrate metabolism of total testicular tissue were extensively reviewed by Free (1970). Utilization of glucose by testicular tissue was demonstrated in different species, and it was shown that intact testicular tissue from adult rats is extremely dependent on glucose supply as compared to 16 other tissues. From studies with testicular tissue containing different germ cell populations, Free (1970) suggested that blood glucose is mainly used by germ cells, while lipids provide the main source of energy for non-germinal testicular cells. The role of amino acids and lipids as sources of energy for testicular cells was not extensively investigated. In contrast, pathways of carbohydrate metabolism in the testis have been amply investigated (review: Free, 1970). However, these investigations have been performed with total testicular tissue which contains cell types with various functions which require different metabolic pathways. Therefore it is difficult to ascribe pathways to individual cell types.

Activities of individual cells can be investigated with autoradiographic studies. It was shown with autoradiography that protein synthesis in pachytene spermatocytes and round spermatids can be stimulated by addition of glucose to testis slices in vitro (Davis, 1969). Based on these studies and the studies mentioned previously in this section, it was suggested that glucose directly stimulated the synthetic activity of pachytene spermatocytes and round spermatids. Preliminary investigations in our laboratory, however, showed only a minor effect of glucose and a

major effect of its metabolites (pyruvate and lactate) on synthetic activities (RNA - and protein synthesis) of isolated spermatocytes and spermatids. Hence, some aspects of carbohydrate metabolism in isolated germ cells (section 4.1) and Sertoli cells (section 4.2) were investigated. The possible dependence of germ cells on carbohydrate metabolism of Sertoli cells induced studies on the effect of hormones on the carbohydrate metabolism of Sertoli cells (section 4.2), and the possible interaction between Sertoli cells and germ cells via intermediates of carbohydrate metabolism (section 4.3).

4.1. Carbohydrate metabolism of isolated germ cells

For studies on the effects of substrate supply on germ cells, measurements of energy-requiring activities like RNA - and protein synthesis seemed an appropriate endpoint. A scheme for the pathways of carbohydrate metabolism in germ cells, based on data presented in sections 4.1.1 and 4.1.2, is presented in Figure 4.1.

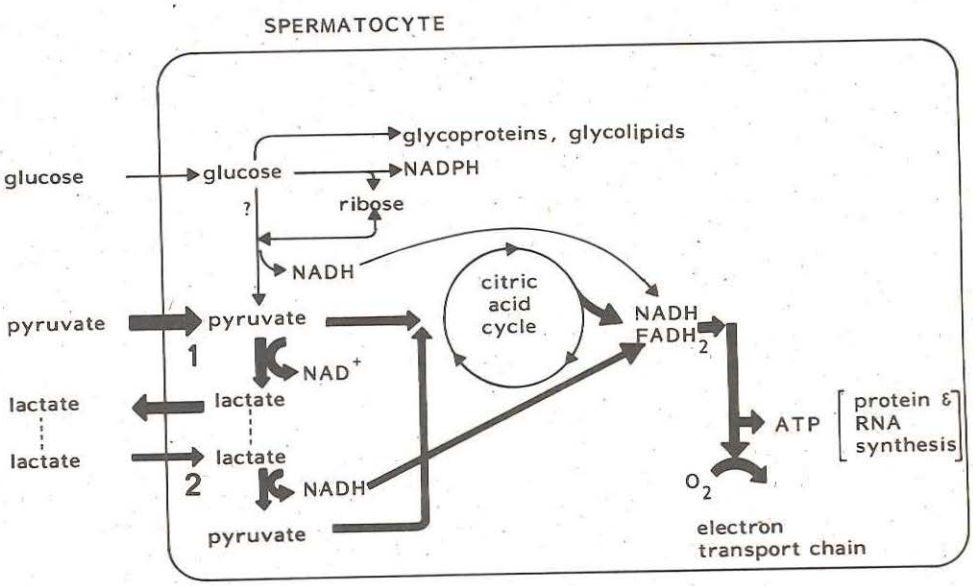


Figure 4.1. Pathways of carbohydrate metabolism in pachytene spermatocytes incubated in the presence of glucose and pyruvate (1) or in the presence of glucose and lactate (2). See section 4.1.

4.1.1. Effect of carbohydrates on synthetic activities of germ cells

Medium, composed in our laboratory for the incubation of isolated germ cells, which contained glucose, pyruvate and lactate, was successfully used to investigate RNA - and protein synthesis in these cells (Grootegoed et al., 1977a,1982c; Appendix Paper I). An indication that glucose was not the most important substrate to support isolated germ cells, was obtained by the removal of pyruvate and lactate from the medium. When observed by phase-contrast microscopy, isolated spermatocytes showed signs of degeneration after incubation for 24 h in medium containing only glucose, also when insulin (134 mU/ml) was added. When pyruvate and/or lactate were present in the medium, the morphology of spermatocytes after 24 h was still very similar to that of freshly isolated cells. Therefore, the effects of pyruvate, lactate and glucose on the synthetic activities of isolated germ cells were further investigated.

Addition of glucose to spermatocytes and round spermatids did only have a small effect on uridine and leucine incorporation, and no effect on oxygen consumption. Addition of lactate in the absence or presence of glucose, however, increased both uridine and leucine incorporation at least five-fold and oxygen consumption two-fold (Appendix Paper II). In cell preparations enriched with pachytene spermatocytes or round spermatids, which were incubated in the presence of glucose, the effect of lactate and pyruvate on protein synthesis was shown to be dose-dependent (Appendix Paper IV). Pyruvate stimulated the leucine incorporation into spermatocytes and spermatids at lower concentrations than lactate. Maximal leucine incorporation was reached at 0.2 mM pyruvate or 2-3 mM lactate (Appendix Paper IV). Addition of lactate together with a high dose of pyruvate did not further increase protein synthesis above the level obtained with pyruvate alone, indicating that both substrates stimulate activities via the same routes (Figure 4.2). These observations strongly suggest that pyruvate and lactate can supply more energy for maintenance of synthetic activities in spermatocytes and spermatids than glucose.

Other investigators partly confirmed and extended our observations. Glucose, fructose, galactose, mannose, ribose, glycerol and acetate had only a small stimulatory effect on ATP levels and protein synthesis in round spermatids (Nakamura & Hall, 1976,1977; Nakamura et al., 1978, 1981a,b; Nakamura & Kato, 1981; Mita & Hall, 1982), whereas lactate had a

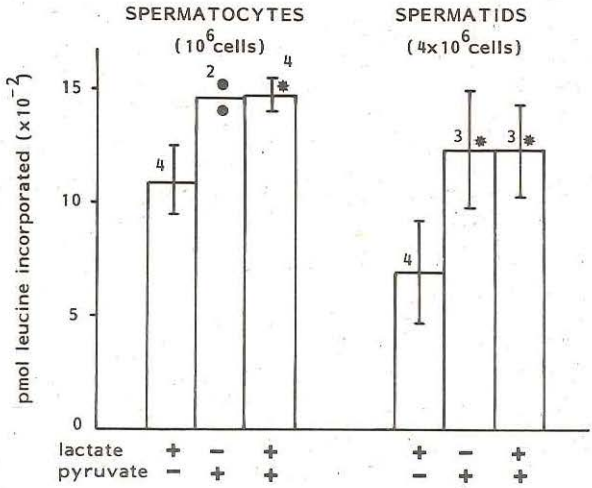


Figure 4.2. Effect of addition of lactate on the incorporation of leucine into isolated germ cells incubated in the presence of 2 mM pyruvate. Leucine incorporation into isolated pachytene spermatocytes or round spermatids, incubated in the presence of lactate (6 mM DL) and/or pyruvate (2 mM), was estimated as described in Appendix Paper II. The number of incubations is stated at each column.
* $p < 0.02$ when compared to the activity in the presence of lactate.

major stimulatory effect on ATP levels, protein synthesis and oxygen consumption in these cells (Nakamura et al., 1981a,b; Nakamura & Kato, 1981). The effect of lactate on protein synthesis was not mediated by a stimulation of transport of amino-acids into the cells (Nakamura et al., 1981b). However, the following observations from these investigators appear contradictory. In contrast to our results, Nakamura & Kato (1981) did not observe an effect of lactate on uridine incorporation into RNA of round spermatids. In addition, although both pyruvate and lactate stimulated protein synthesis, only lactate was effective in increasing the ATP levels in spermatids (Nakamura et al., 1981a; Mita & Hall, 1982).

In our studies incubation of isolated germ cells in the absence of pyruvate and lactate had a rapid effect on cellular activities, as indicated by the decrease in protein synthesis in spermatocytes (Figure 4.3), which is supported by the depletion of ATP in spermatids observed by Nakamura et al. (1981a) after 30 min. After a few hours of incubation in the absence of p & l irreversible changes were induced (Appendix Paper II).

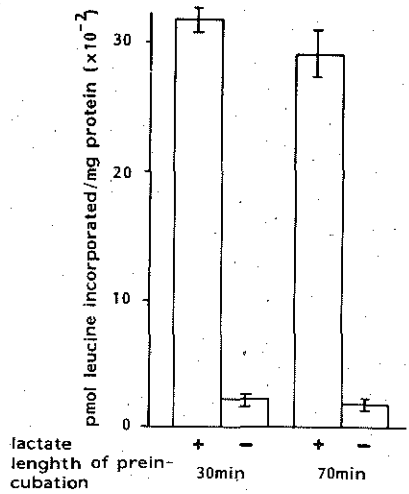


Figure 4.3. Rapid effect of p & l depletion on the leucine incorporation into pachytene spermatocytes.
Cell fractions enriched with pachytene spermatocytes were incubated in incubation medium without additions or in the presence of 6 mM DL-lactate. After 30 or 70 min 0.6 µCi (^{14}C)leucine was added, followed by an incubation of 2 h. Subsequently, cold 0.9% NaCl (w/v), containing 3 mM leucine, was added and the cells were washed thrice in this solution by centrifugation for 7 min at 200 g at 4°C. Next, the cells were collected by centrifugation at 600 g and lysed in 0.5% SDS during 10 min in ice. Macromolecules were precipitated in cold 10% trichloroacetic acid, followed by three washings in 10% TCA by centrifugation for 5 min at 700 g at 4°C. Finally, the pellets were washed with 96% ethanol, centrifuged for 10 min at 1500 g at 4°C, dried and solubilized in 1 N NaOH. Part of this solution was counted for radioactivity and part was used for protein estimation (Lowry et al., 1951). Mean values and range of duplicate incubations are given. The results indicate that after 30 min of incubation in the absence of p & l but in the presence of glucose, no substrate remained in the cell which could maintain protein synthesis during the subsequent period.

Combined morphological and biochemical studies of isolated germ cell mitochondria indicate that the typical condensed mitochondria, present in late pachytene spermatocytes and round spermatids, have a high oxidative capacity (Setchell, 1978; De Martino et al., 1979). Moreover, in early round spermatids, mitochondria are localized close to the cell surface (Fawcett, 1974). These observations suggest that pachytene spermatocytes and round spermatids are equipped to oxidize exogenous p & l at a high rate.

In conclusion, the presence of p & l appears to be essential for the maintenance of synthetic activities in isolated germ cells. The com-

bined biochemical and morphological observations indicated that decreased synthetic activities are correlated with a decrease in survival time of the germ cells.

4.1.2. Non-energy generating pathways of carbohydrate metabolism in germ cells

Glucose was shown to be inadequate in maintaining synthetic activities in isolated spermatocytes and spermatids in short-term experiments. However, the presence of glucose might be required in long-term incubations, as a substrate for the hexose monophosphate shunt, for the supply of intermediates, such as ribose, for RNA synthesis, and to keep a proper NADPH/$NADP^+$ balance, or glucose may be used for glycoprotein or glycolipid synthesis. It has been shown that RNA synthesis (review: Bellvé, 1979) and fucosylation and glycosylation of proteins take place in late pachytene spermatocytes and spermatids (Grootegoed et al., 1982c; Letts et al., 1978).

After incubation of isolated germ cells in the presence of pyruvate, lactate was found in the incubation medium, and the amount of lactate in the medium was increased even in the presence of added lactate and irrespective of the presence of glucose (Table 4.1). At least 50% of the consumed pyruvate was converted to lactate. After incubation of germ cells in the presence of lactate, no pyruvate was found in the incubation medium in our experiments (Table 4.1), which is in contrast to the results of Mita & Hall (1982), who observed secretion of pyruvate by round spermatids.

The conversion of pyruvate to lactate reduces the amount of NADH available for ATP generation in the electron transport chain, firstly by diverting pyruvate from oxidation in the Krebs cycle, which would generate NADH, and secondly because NADH is oxidized concomitant with pyruvate reduction. Therefore, the conversion of pyruvate to lactate appears to be a waste for the cell in terms of energy supply, but it could play a role to maintain a proper ratio between NAD^+ and NADH in the cells. In spite of the apparent waste of energy-yielding substrates when pyruvate is added, germ cells acquire maximal synthetic activity at a pyruvate concentration which is 10-fold lower than the lactate concentration required (Appendix Paper IV). Therefore, the effect of pyruvate on isolated germ cells might be

Table 4.1. p & l consumption and secretion by isolated germ cells.

incubation conditions	µmol p & l./10^6 cells/24 h			
	pyruvate consumed	lactate secreted	lactate consumed	pyruvate secreted
glucose + pyruvate	3.52 ± 0.42 (3)	2.08 ± 0.40 (3)	-	-
pyruvate	3.80 ± 0.26 (2)	2.40 ± 0.04 (2)	-	-
glucose + pyruvate + lactate	3.24 ± 0.26 (3)	1.90 ± 0.32 (3)	-	-
glucose + lactate	-	-	0.70 ± 0.28 (4)	0 (2)

Pachytene spermatocytes (0.5 x 10^6 cells) were incubated in 2 ml incubation medium in the presence of pyruvate (2 mM), lactate (6 mM DL) and/or glucose (3.3 mM) or combinations of these substrates. After 24 h the amounts of pyruvate and L-lactate in the media were estimated. Results are presented as means ± S.D. The numbers between brackets indicate the number of cell preparations used.

The results indicate that the consumption of pyruvate and the amount of pyruvate converted to and secreted as lactate were similar in the presence or absence of added glucose or lactate. No secretion of pyruvate was observed after incubation of spermatocytes in the presence of lactate.

two-fold, viz. firstly the effect of pyruvate as a source of energy, and secondly an effect on the redox balance in the cells by pyruvate reduction.

4.1.3. Possible other energy sources for spermatocytes and spermatids

No depots of energy-rich substrates, like glycogen or lipids, have been reported for pachytene spermatocytes or round spermatids. The apparent absence of energy stores is confirmed by the rapid effect of triose depletion on the synthetic activities of these germ cells (section 4.1.1; Appendix Paper II).

In vivo, exogenous substrates for germ cells could be supplied via the fluid which surrounds the germ cells, but the chemical composition of this fluid is largely unknown. More is known about the fluid in the rete testis, where the ends of the tubules meet. It must be kept in mind that the composition of the latter fluid may differ from the composition of the fluid which is closer to the germ cells, as has already been shown for the ionic composition (Setchell, 1978). Moreover, the meaning of high concentrations of substrates in rete testis fluid is by no means unequivocal; it may reflect either that these substrates have to be available to germ cells or that they are not used by the germ cells and are being removed as waste.

In many species the concentration of myoinositol in the rete testis fluid is very high in contrast to the concentration of glucose and fructose, which are hardly detectable (Setchell, 1978). Myoinositol has been shown to be an important nutrient for mammalian cells in culture (Eagle et al., 1957). However, myoinositol did not increase the ATP level in round spermatids (Mita & Hall, 1982). Rete testis fluid from rats contains high amounts of the amino-acids: aspartic acid, alanine, glycine, proline and lysine, as compared to plasma. In some other species glutamic acid is increased instead of proline and lysine (Setchell, 1978). These amino-acids might be used by the germ cells as an energy source or as substrates for purine and pyrimidine synthesis. However, the amino-acids present in the incubation medium were not sufficient to maintain synthetic activities in isolated germ cells (section 4.1.1).

Little is known about lipids as substrates for germ cells. The specific activity of carnitine acyltransferase, an enzyme involved in the translocation of fatty acid across the mitochondrial membrane, was low in

spermatogonia and high in pachytene and diplotene spermatocytes (Vernon et al., 1971). However, this enzyme is required in fatty acid synthesis as well as fatty acid oxidation and it was shown recently that purified mouse pachytene spermatocytes and round spermatids synthesize cholesterol and dolichol (Potter et al., 1981). From experiments with testicular cell suspensions from rats of different ages, it was concluded that spermatogonia were active in palmitate oxidation, but the activity of spermatocytes and spermatids appeared to be less (Lin & Fritz, 1972). In conclusion, up to now there are no indications that other substrates than p & l are important energy sources for pachytene spermatocytes and round spermatids.

4.1.4. Presence of germ cell-specific enzymes

It is striking that several isozymes of enzymes which catalyze pathways involved in carbohydrate metabolism, are located in germ cells and not in any other cell type. Some of these enzymes are involved in lactate and/or pyruvate oxidation, viz. lactate dehydrogenase-C_4 (LDH-X) and cytochrome c_t. These testis-specific isozymes emerge in mid-late pachytene spermatocytes and remain present in more mature germ cells (Hennig, 1975; Goldberg et al., 1977; Hintz & Goldberg, 1977; Meistrich et al., 1977; Wheat et al., 1977). LDH-C_4 was shown to catalyze preferentially lactate oxidation (review: Goldberg, 1977). It has been suggested that LDH-C_4 is involved in a shuttle for transport of reducing equivalents across the mitochondrial membrane (review: Blanco, 1980). Nothing is known about a specific kinetic property of cytochrome c_t, although the amino-acid sequence has been elucidated (Hennig, 1975). In addition, several testis-specific glycolytic isozymes, hexokinase (reviews: Gomes & VandeMark, 1974; Goldberg, 1977), phosphoglycerate kinase (PGK-2) (Van de Berg et al., 1973,1976,1981; Kramer, 1981), enolase (Edwards et al., 1981) and glucose phosphate isomerase (Buehr & McLaren, 1981) are known, which are more abundant in spermatozoa than in developing germ cells.

Another germ cell-specific property is the inactivation of the sex chromosomes throughout the meiotic prophase, while the autosomes are transcriptionally active (Monesi, 1965). Two enzymes involved in glucose metabolism, viz. glucose 6-phosphate dehydrogenase, which catalyzes the first reaction of the hexose monophosphate shunt, and an isozyme of the glycolytic enzyme phosphoglycerate kinase (PGK-1), are coded for by the

X-chromosome. This may be the cause of the absence or low activity of these enzymes in elongating spermatids and spermatozoa (Van de Berg et al., 1973, 1976,1981; Erickson, 1976; Brock, 1977; Kramer, 1981). It must be kept in mind, however, that germ cells also may contain non-testis-specific isozymic forms or autosomally linked forms of the enzymes mentioned in this section. Hence, the understanding of the importance of the testis-specific isozymes awaits elucidation of their specific kinetic properties.

4.1.5. Effect of temperature on isolated germ cells

Increase of the temperature of the testis from 32° to 37°C is known to induce degeneration of pachytene spermatocytes and more mature germ cells. This may be caused by disturbances in carbohydrate metabolism, because addition of glucose during the incubation of testicular tissue prevented the decrease in protein synthesis, which was observed at temperatures above 32°C (Davis, 1969).

Isolated, partly purified pachytene spermatocytes and spermatids incubated at 32° or 37°C showed increased membrane damage at the higher temperature (Lee, 1974). In isolated round spermatids increase in temperature caused a decrease of protein synthesis (possibly by an effect on the initiation of protein synthesis or by a change in availability of messenger-RNA) and a decrease of transport of hexoses and amino-acids. The effect of temperature on this transport was reversible, which was interpreted as an indication that cell death was not causing the phenomena observed (Nakamura et al., 1978; Nakamura & Hall, 1978; Hall et al., 1981; Hall & Nakamura, 1981). None of these effects were found on spermatocytes, although it was shown that these cells are also affected by cryptorchidism in mature animals (Davis, 1969; Setchell, 1978; Rommerts et al., 1980).

These effects of temperature increase on isolated germ cells were observed during incubations in the absence of pyruvate and lactate and it remains to be demonstrated whether similar changes are induced in the presence of proper energy yielding substrates.

4.2. Carbohydrate metabolism of isolated Sertoli cells

4.2.1. Glucose metabolism of Sertoli cells

We have investigated whether Sertoli cells could supply the p & l required by pachytene spermatocytes and round spermatids. A scheme for the pathways of carbohydrate metabolism in Sertoli cells, based on data presented in this section, is shown in Figure 4.4.

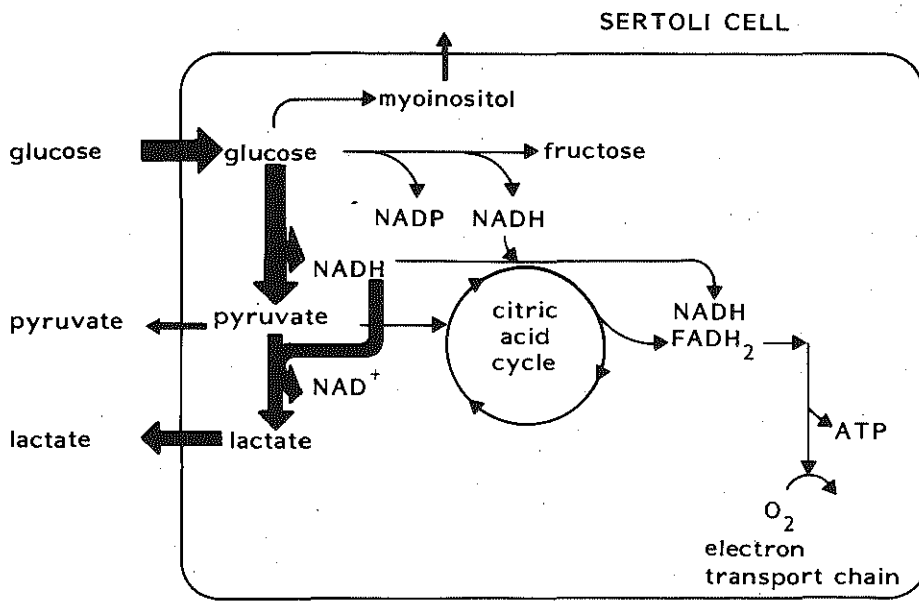


Figure 4.4. Pathways of carbohydrate metabolism in Sertoli cells incubated for 24 h in the presence of glucose. See section 4.2.1.

Both pyruvate and lactate were secreted by Sertoli cells from 4-week-old rats (Appendix Papers III, IV). Approximately 4 times more lactate than pyruvate accumulated in the medium. The amount of lactate, present in the medium of Sertoli cells from prenatally irradiated rats or from intact rats, increased with increasing exogenous concentrations of glucose (Appendix Paper III), indicating that exogenous glucose is converted to lactate.

Sertoli cells from 20 day old intact rats were reported to convert maximally 2.9% of the glucose utilized to CO_2, while 95.8% was converted to anionic compounds, mainly to lactate, during incubation for 60 min (Robinson & Fritz, 1981). In our studies, during incubation of Sertoli cells from 4-week-old rats for 24 h approximately 50% of the glucose utilized accumulated as lactate (Appendix Paper III). These observations may indicate that, when the p & l concentration in the medium increases during prolonged incubation, Sertoli cells utilize glucose in other pathways not involved in p & l secretion.

It was shown previously that Sertoli cells in vitro can convert glucose to myoinositol (Robinson & Fritz, 1979) which is present in high concentrations in rete testis fluid (Setchell, 1978). However, the quantitative importance of this pathway in the total rate of glucose utilization by Sertoli cells seems small (Robinson & Fritz, 1981). Another minor glucose-consuming pathway in Sertoli cells might be the conversion of glucose to sorbitol catalyzed by aldose reductase, which was recently shown to be present in Sertoli cells and not in germ cells (Ludvigson et al., 1982). Together with sorbitol dehydrogenase, aldose reductase constitutes the polyol pathway, which has a net production of NADP and NADH, and of fructose when glucose is the substrate (Metzler, 1977; Ludvigson & Sorenson, 1980). Intravenous infusion of radioactively labelled glucose in rams results in the appearance of radioactively labelled amino-acids in rete testis fluid (Setchell, 1975). It is not known whether this conversion takes place in Sertoli cells.

In conclusion, Sertoli cells from intact as well as from prenatally irradiated rats appear to have a large capacity to convert glucose to p & l, which are secreted by the cells, and little p & l appear to be used as energy source by Sertoli cells. However, in vivo a high extracellular p & l concentration or a change in the ratio between NAD and NADH may influence the rate of secretion of pyruvate and lactate by Sertoli cells, and other pathways of glucose metabolism may become more important.

4.2.2. Non-carbohydrate substrates and energy metabolism of Sertoli cells

It was suggested previously, based on investigations with total testicular tissue, that non-germinal cells in the testis are dependent on lipid oxidation for energy supply (Free, 1970). Lipid droplets are often observed in the cytoplasm of Sertoli cells. In vitro as well as in vivo the amount of lipid stored in Sertoli cells increases parallel to germ cell degeneration (Fawcett, 1975), suggesting that germ cells which have been phagocytized are an energy source for Sertoli cells. However, increase in lipid material was also shown to occur in Sertoli cells in prenatally irradiated (germ cell-depleted) testes as a result of cryptorchidism (Bergh, 1981). When isolated Sertoli cells were incubated for one day in the absence of glucose and p & 1 followed by incubation in the presence of glucose, the cells retained the property to migrate on the bottom of the Petri dishes and retained their p & 1-secreting capacity. Sertoli cells may have used lipids as energy substrates to survive one day of glucose depletion.

A possible role for amino acids as additional energy substrates for Sertoli cells is suggested by a high rate of branched-chain amino acid oxidation by Sertoli cells in vitro (data not shown) (Grootegoed et al.).

In vivo Sertoli cells may have access to all substrates present in the interstitial lymph fluid and the amount of substrates available to Sertoli cells can be regulated by the testicular blood flow and the permeability of the testicular capillaries. In conclusion, non-carbohydrate substrates may be a major source of energy for Sertoli cells, but to support this conclusion, further investigations are required.

4.2.3. Direct effects of hormones on Sertoli cells

FSH

It is well established now that follicle-stimulating hormone (FSH) can stimulate many Sertoli cell activities in vitro and in vivo (recent reviews: Means et al., 1976,1978,1980; Fritz, 1978; Davies, 1981; Purvis & Hansson, 1981). This FSH stimulation is age-dependent and stimulation of intracellular events decreases in rats above 20 days of age (Fritz, 1978; Steinberger et al., 1978; Means et al., 1980), whereas stimulation of oestradiol secretion decreases above 5 days of age (Dorrington et al.,

1978). The p & 1 secretion by Sertoli cells from 3, 4, 5 and 6-week-old prenatally irradiated rats can be stimulated by FSH. However, the relative response appeared to decrease with age concurrent with an increasing basal level of the p & 1 secretion (Appendix Paper IV). Because stimulation by FSH was most effective in Sertoli cells from 4-week-old rats, cells of this age were further investigated. FSH stimulation of both pyruvate and lactate secretion by Sertoli cells was dose-dependent and half maximal stimulation was obtained with approximately 10 ng FSH-S13/ml (Appendix Paper IV), which is a lower ED_{50} than reported by others (Mita et al., 1982) (Table 4.2). Other known ED_{50}'s of FSH on Sertoli cell activities in vitro are summarized in Table 4.2. FSH stimulated the p & 1 secretion in tubules from 4-week-old intact rats three-fold.

Testosterone

Testosterone can support spermatogenesis in adult animals. Therefore, the effect of testosterone on the p & 1 secretion was investigated in Sertoli cells from 4- and 6-week-old rats. No effect of testosterone on p & 1 secretion was observed at both ages either in the presence or absence of FSH (Appendix Papers III, IV). This might be caused by the presence of a low basal level of testosterone, synthesized by a few contaminating Leydig cells in the Sertoli cell preparation. Effects of testosterone on Sertoli cell activities in vitro shown previously, are the secretion of proteins (Rommerts et al., 1978), such as inhibin (Franchimont et al., 1980) and ABP (Louis & Fritz, 1977), and the activity of RNA polymerase (Lamb et al., 1981), which were stimulated by testosterone in Sertoli cells from 19-25-day-old rats (Table 4.2).

Insulin

Insulin was found to stimulate the p & 1 secretion by Sertoli cells from 4-week-old rats two-fold, whereas the stimulation by FSH in these experiments was four-fold (Appendix Paper IV). The effects of insulin and FSH were not additive, although the Sertoli cells were capable to secrete 6 times the control level of p & 1 , as is shown in incubations in the presence of dibutyryl-cyclic AMP (bt_2cAMP) plus a phosphodiesterase inhibitor (MIX). In contrast to these results, the FSH-stimulated rate of transferrin secretion by Sertoli cells was further enhanced by insulin (Skinner & Griswold, 1982).

Table 4.2. Comparison of the doses of FSH or testosterone necessary for stimulation of different Sertoli cell activities in vitro.

Sertoli cell activity	ED_{50} (ng/ml)	age (days)	incubation period (hours)	Reference
FSH				
ABP secretion	3.5	20	72-144*	Louis & Fritz, 1979
Protein kinase inhibitor	100	16 sterile	0- 72*	Tash et al., 1980
Pyruvate/lactate secretion	150	28 sterile	24- 48*	Appendix Paper IV
Sulfoprotein secretion	162 (MIX)	20	48-120	Elkington & Fritz, 1980
Plasminogen activator secretion	180/460	20	24-?	Lacroix & Fritz, 1982
DNA synthesis	219	20	72- 96	Fritz et al., 1978
Testosterone aromatization	288	18-20	24- 48	Dorrington et al., 1978
Phosphodiesterase	300	19	168-192	Verhoeven et al., 1981
Lactate secretion	635	16	48- 54	Mita et al., 1982
Intracellular cAMP	815	20	48- 49	Fritz et al., 1978
Intracellular cAMP	1150-3450	18-24-33	96- 96.5	Steinberger et al., 1978
Phosphodiesterase	200 rat FSH-B1	15	120-144	Conti et al., 1981
Testosterone				
ABP secretion	1.2	20	72-168*	Louis & Fritz, 1979
Inhibin secretion	144	19	144-168	Franchimont et al., 1980
RNA polymerase	6056	25	120-120.25	Lamb et al., 1981

The different ovine FSH preparations used were converted to FSH-S1 Units. 1 ng Sairam FSH-S1554 = 45 ng FSH-S11 (Louis & Fritz, 1979); FSH-S11 = 1.15 FSH-S1; FSH-S12 = 1.25 FSH-S1; FSH S13 = 15 FSH-S1.
* Hormone present from the time of plating.

TSH

Recently, thyroid-stimulating hormone (TSH) was shown to stimulate the activity of Sertoli cells from 16-day-old rats, as expressed in an increased secretion of ABP and plasminogen activator (Hutson & Stocco, 1981).

In summary, Sertoli cells in vitro can be stimulated by FSH, testosterone, insulin and TSH. The p & 1 secretion by Sertoli cells was increased by FSH and insulin, but not by testosterone.

4.2.4. Effect of incubation conditions on Sertoli cells

We have observed that the p & 1 secretion by Sertoli cells from 4-and 5-week-old rats was higher during the first 24 h of incubation than during the following 24 h (24-48 h) (Figure 4.5). The FSH-stimulated p & 1 secretion by these cells decreased also during incubation, but at a lower rate. Possibly the effect of FSH stimulation in vivo is temporarily retained in vitro. Hence, the ratio stimulated/unstimulated p & 1 secretion

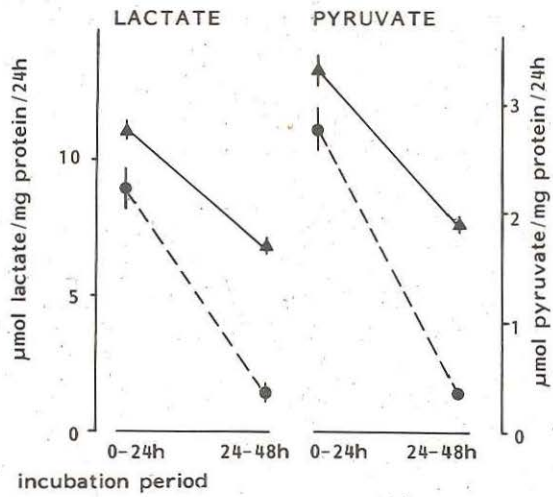


Figure 4.5. Decrease of the amount of p & 1 secreted by Sertoli cells during incubation.
Sertoli cells from prenatally irradiated rats were isolated at 4 weeks of age. The cells were isolated and incubated as described in Materials and Methods in the absence (●---●) or presence of NIH-FSH-S13 (0.5 µg/ml) and testosterone (200 ng/ml) (▲——▲). The media of the first and the second day of incubation were collected separately, the amounts of pyruvate and lactate were estimated and expressed per mg protein present at the end of the incubation. Mean and range of duplicate incubations are given.

increased during incubation. Other activities of Sertoli cells have been shown to change during incubation, viz. ABP - and transferrin secretion by Sertoli cells from 20 day old rats decreased (Louis & Fritz, 1977; Rommerts et al., 1978; Gianetto & Griswold, 1979; Karl & Griswold, 1980; Skinner & Griswold, 1982), basal cyclic AMP levels in Sertoli cells from 33 day old rats and FSH-stimulated oestradiol secretion by Sertoli cells from 20 day old rats increased (Rommerts et al., 1978; Steinberger et al., 1978) and protein synthesis changed (Chapter 3, Appendix Paper I). Therefore, care must be taken in extrapolating *in vitro* results directly to the *in vivo* situation.

4.3. Metabolic dependence of germ cells on Sertoli cells

In the previous sections some aspects of carbohydrate metabolism by isolated germ cells and isolated Sertoli cells have been reported. In this section it will be considered whether and to which extent the activities of germ cells are influenced by Sertoli cells.

4.3.1. Effect of glucose depletion on the activity and survival of germ cells

Isolated germ cells were shown to require p & l as substrates for maintenance of synthetic activities (section 4.1.1). We observed also, that glucose supply to Sertoli cells is correlated with the p & l secretion by Sertoli cells (sections 4.2.1). Therefore, we have investigated the effect of glucose on the activity and survival of germ cells incubated in the presence of Sertoli cells.

Fragments of seminiferous tubules from 4½-week-old rats, containing germ cells up to and including round spermatids, were incubated without glucose for 24 h followed by incubation in the presence of glucose. After spreading of the tubule fragments, pachytene spermatocytes and round spermatids were virtually absent from the cultures, whereas in control tubules incubated in the continuous presence of glucose pachytene spermatocytes and round spermatids were still present. Many early spermatocytes and spermatogonia appeared to remain present under both conditions. The latter observation would agree with autoradiographic studies of slices of testis tissue incubated in the absence or in the presence of glucose. The

early spermatocytes and spermatogonia A in these slices retained a high level of leucine incorporation in the absence of glucose (Davis, 1969) and therefore, these cells appear less dependent on supply of glucose or its metabolites than the more mature germ cells. The degeneration of the majority of the germ cells in fragments of seminiferous tubules incubated in the absence of glucose was confirmed by quantitative cytofluorometric measurement of the cell types present in the incubations (Appendix Paper III).

For measurement of the synthetic activity of germ cells in the presence of Sertoli cells, a homogeneous population of germ cells was co-incubated with a monolayer of Sertoli cells. The incorporation of uridine into pachytene spermatocytes co-incubated with Sertoli cells in increasing glucose concentrations increased concomitant with the lactate secretion by Sertoli cells (Appendix Paper III). Spermatocytes, incubated under comparable conditions in the absence of Sertoli cells but in the presence of lactate and glucose, showed the same uridine incorporations as spermatocytes co-incubated with Sertoli cells in the presence of glucose. Because it was demonstrated that glucose has only a small effect on spermatocytes (section 4.1.1), the results suggest that glucose influenced germ cells via p & l secretion from Sertoli cells. Only lactate was measured, but pyruvate was probably also involved.

In conclusion, survival of pachytene spermatocytes and round spermatids, and RNA synthesis in pachytene spermatocytes, could be regulated by glucose in the presence of Sertoli cells. This regulation was accompanied by changes in the p & l secretion by Sertoli cells, suggesting that germ cell activity can be regulated by p & l secretion from Sertoli cells.

Possibly, glucose depletion in testicular tissue is also involved in the effects of cryptorchidism on germ cell activities. The blood flow in the cryptorchid testis is either unchanged or slightly increased as compared to the scrotal testis (Setchell, 1978). Therefore, the amount of glucose available to the Sertoli cells in the cryptorchid testis would not be increased and the amount of p & l secreted would remain rather constant during temperature elevation. Hence, an increased demand for energy-rich substrates by germ cells caused by the higher temperature may not be compensated by an increased supply and this may result in degeneration of pachytene spermatocytes and round spermatids (see also section 4.1.5).

4.3.2. Effect of secretion products from Sertoli cells on germ cells

It cannot be excluded from the experiments described in the previous section, that the stimulation of germ cell activities was mediated by membrane contacts between the germ cells and the Sertoli cells. Therefore, germ cells were incubated in medium which was conditioned by Sertoli cells (Appendix Paper IV). Isolated pachytene spermatocytes and round spermatids incubated in the conditioned medium incorporated significantly higher amounts of leucine than cells incubated in fresh medium. The total leucine incorporation into cells incubated in the conditioned medium was similar to the incorporation into cells, which had been incubated in fresh medium containing the same concentration of pyruvate and lactate as the conditioned medium. These observations confirm that secretion products from Sertoli cells can stimulate activities of germ cells and p & l seem to be the major secretion products from hormone-stimulated Sertoli cells which affect the synthetic activity of germ cells.

4.3.3. Indirect effects of hormones on germ cells via Sertoli cells

With cytofluorometric measurements we have found, that more primary spermatocytes appeared to survive in fragments of seminiferous tubules incubated in the absence of hormones than in tubules of hypophysectomized rats (Figure 4.6). The relative number of spermatocytes in these incubations of tubule fragments was virtually equal to the number in tubules of intact rats (Figure 4.6). Therefore, incubation of tubule fragments under these conditions appears close to optimal for the survival of spermatocytes for a few days. This may explain why no effect of FSH and/or testosterone was observed on the synthetic activities of spermatocytes co-incubated with Sertoli cells, or on survival of spermatocytes in incubations of fragments of seminiferous tubules (results not shown). It may be possible that some nutrients (glucose, amino acids etc.) are available at a higher concentration *in vitro* than *in vivo*. Under these conditions unstimulated Sertoli cells may produce sufficient p & l for germ cells to survive. To demonstrate an effect of hormones on germ cells *in vitro*, imitation of the effect of hypophysectomy may require a change in incubation conditions of seminiferous tubules, so that germ cells degenerate *in vitro* in the absence of hormones.

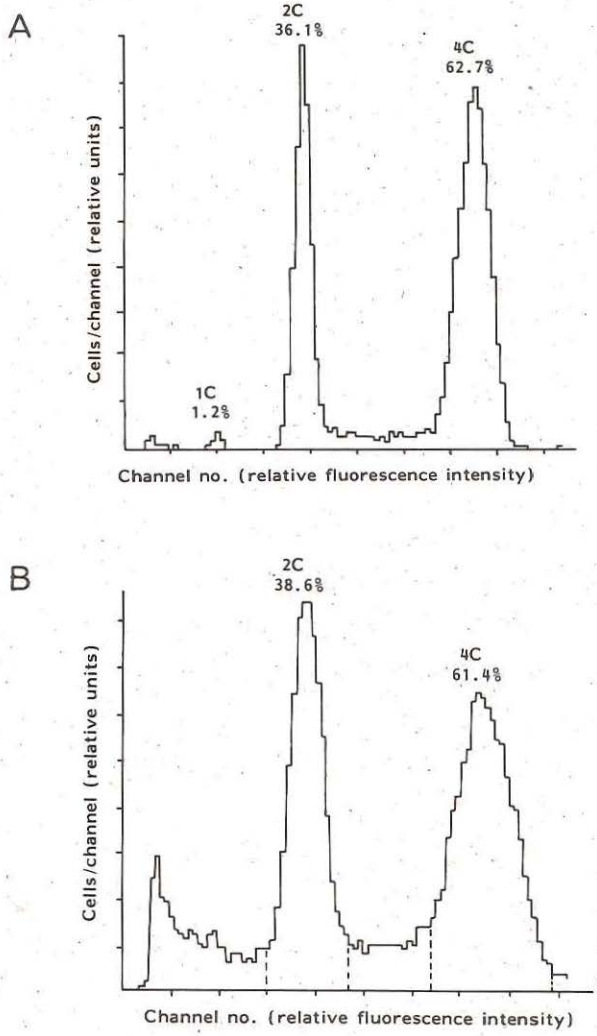
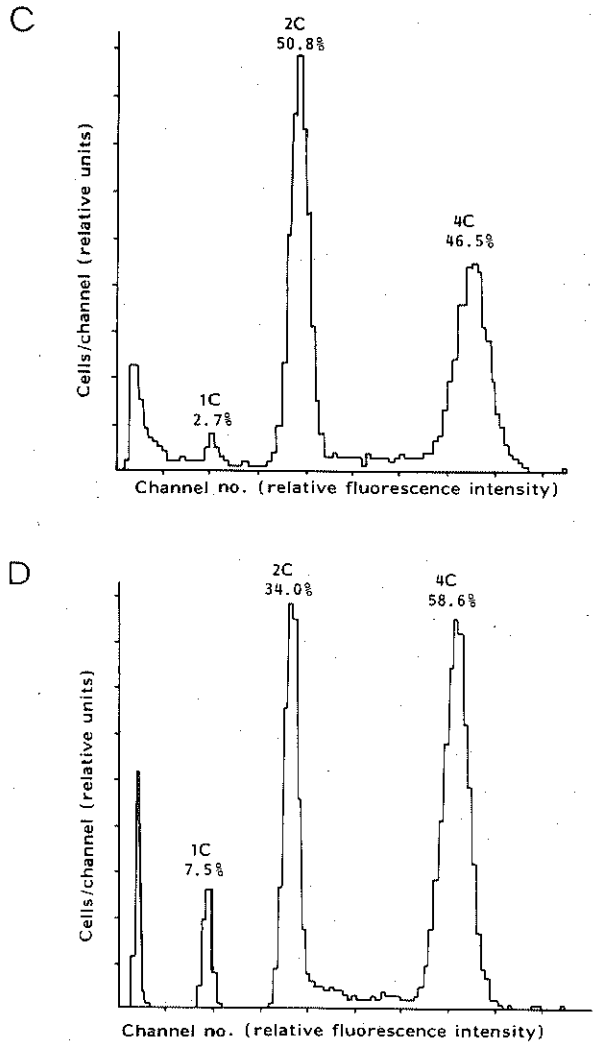


Figure 4.6. Effect of incubation on the composition of the cell population in seminiferous tubules isolated from 25-day-old rats and comparison with the effect of hypophysectomy.
Cell preparations were obtained from seminiferous tubules of 25-day-old intact rats immediately after isolation (A) or following incubation of the tubules for 4 days in the absence of hormones (B), of 29-day-old rats hypophysectomized at 25 days of age (C) and of 29-day-old intact rats (D). The number of cells and their DNA content were measured by DNA flow cytometry. The quantity of cells containing 1C, 2C or 4C DNA (see Fig. 2.2) was calculated as percentage of the total number of cells. For comparison, the number of germ cells is expressed relative to the number of Sertoli cells.
The relative number of primary spermatocytes remained virtually constant in intact rats of 25 and 29 days of age (4C/2C = 1.7) and in tubule frag-



ments incubated for 4 days (4C/2C = 1.6). In the hypophysectomized rats the relative number of spermatocytes was decreased after 4 days (4C/2C = 0.9). Some spermatids had developed in intact rats of 29 days old (1C/2C = 0.2 compared to < 0.1 in intact 25 day old rats). No spermatid development was observed in the hypophysectomized rats (1C/2C < 0.1).
These results indicate that germ cell development stops and degeneration of pachytene spermatocytes occurs after hypophysectomy of immature rats. In contrast, no pronounced reduction of pachytene spermatocytes occurred in fragments of seminiferous tubules incubated in the absence of hormones. Hence, the present incubation conditions appear close to optimal with respect to survival of spermatocytes for a few days, and more favourable for survival of these germ cells than the conditions in vivo in seminiferous tubules of hypophysectomized rats.

Chapter 5

GENERAL DISCUSSION

5.1. Changes in carbohydrate metabolism during development of germ cells

The studies on carbohydrate metabolism of germ cells described in this thesis concentrated on pachytene spermatocytes and round spermatids. In this section some information about the effects of trioses and hexoses on other stages of germ cell development will be considered, starting with the earliest stage (Figure 5.1).

It has been shown that primordial germ cells, taken from the gonadal ridges of 15 day-old mouse fetuses, are 10 times more active in pyruvate oxidation than in glucose oxidation (Brinster & Harstad, 1977). This pattern of substrate preference appears to be similar to that of pachytene spermatocytes and round spermatids. However, spermatogonia and early spermatocytes, stages interposed between the primordial germ cell and the pachytene spermatocyte stage, appear less dependent on carbohydrate supply than the more mature germ cells, because spermatogonia and early spermatocytes survived in seminiferous tubules incubated in the absence of glucose, whereas pachytene spermatocytes and round spermatids degenerated (section 4.3.1). Spermatogonia may oxidize palmitate and acetoacetate at a higher rate than pachytene spermatocytes and spermatids, whereas in these experiments spermatogonia appeared to be less active in pyruvate oxidation than the more mature germ cells and thus lipids may be the substrates preferred by spermatogonia (Lin & Fritz, 1972).

Preliminary results indicate that elongated spermatids, in contrast to the preceding stage of round spermatids, maintain their ATP level with glucose as well as with lactate as substrates (Le Gac et al., 1982). Elongated spermatids develop into testicular spermatozoa, which, during their passage through the epididymis, acquire the capacity to be motile and to fertilize. Although development from testicular to ejaculated spermatozoa is accompanied by metabolic changes (Voglmayr, 1975), little is known about the effects of carbohydrates on energy-consuming processes in this stage. In rats, spermatozoa from the cauda epididymis maintained their motility and energy charge for 60 min in the presence of pyruvate and lac-

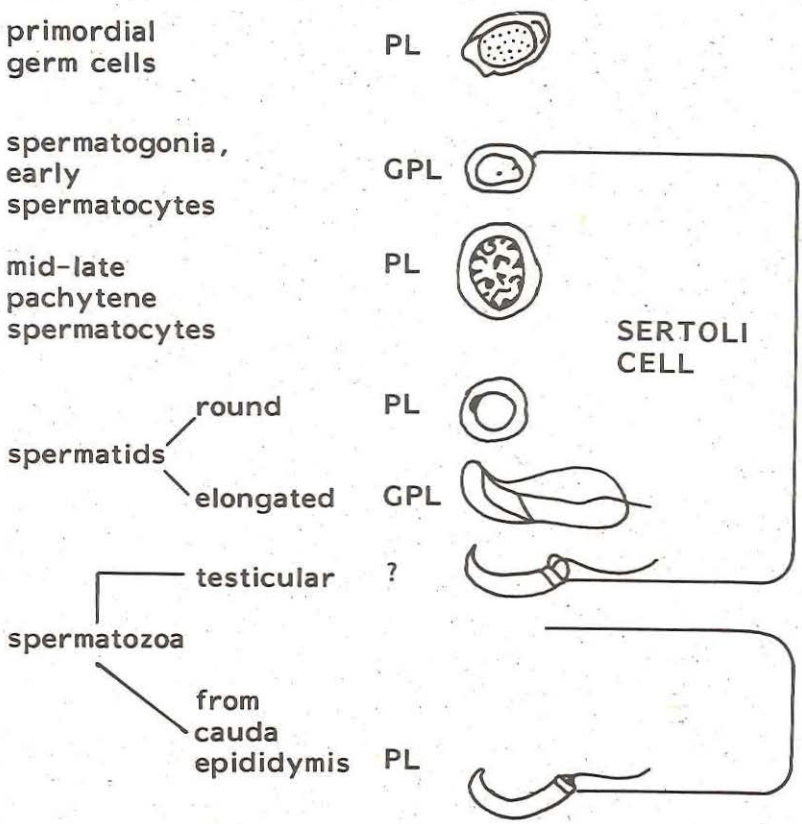


Figure 5.1. Carbohydrates as energy-yielding substrates in different stages of germ cell development.

Preferred substrates: PL - pyruvate and/or lactate
G - glucose
GPL - glucose and/or pyruvate and/or lactate

tate, but not in the presence of glucose (Ford & Harrison, 1981). However, it has been reported that in several species, including man, the motility of ejaculated spermatozoa can be enhanced by hexoses (Mann & Lutwak-Mann, 1981). Many other substrates, e.g. lactate, pyruvate and amino acids, also can stimulate motility, but marked species differences exist (Mann & Lutwak-Mann, 1981). Similar to rat spermatocytes and spermatids (section 4.1.2), spermatozoa from boar and bull metabolize 50% or more of the pyruvate taken up to lactate concomitant with pyruvate oxidation (Mann & Lutwak-Mann, 1981). It is apparent from this information that some differences in carbohydrate dependence occur in the different stages of germ cell development.

The substrate supply to spermatogonia, which are located outside the blood-testis barrier, may be less controlled by Sertoli cells than the supply to pachytene spermatocytes and round spermatids at the luminal side of this barrier, and spermatogonia may have the ability to use a variety of substrates. However, it is unlikely that a dramatic developmental shift in metabolic pattern occurs during the transformation of spermatogonia to pachytene spermatocytes, because spermatocytes resemble primordial germ cells with respect to a high rate of pyruvate oxidation. In 15 day-old fetuses, the male primordial germ cells are surrounded by supporting cells (precursors of Sertoli cells), although no blood-testis barrier exists at this age (Kluin & de Rooij, 1981) and possibly these supporting cells supply p & l to the primordial germ cells, like Sertoli cells to the differentiating germ cells. A decrease in p & l dependence and an increase in glucose utilization appears to occur when round spermatids develop into ejaculated spermatozoa. Concomitantly, the isozymic pattern of some glycolytic enzymes changed (section 4.1.4). The effect of pyruvate and lactate on rat spermatozoa from the cauda epididymis may indicate that elongated spermatids do not loose their dependence on pyruvate and lactate. On the other hand, the relative independence of elongated spermatids on p & l could suggest that an increase in glucose utilization occurs during spermatogenesis.

When the dependence of germ cells on p & l or other substrates changes during spermatogenesis, possible effects of hormones on the availability of substrates may have different effects at different stages of development. The present knowledge of the metabolic requirements of spermatogonia, early spermatocytes and elongated spermatids

suggests that these cells would be less affected by a decrease in p & l secretion by Sertoli cells than pachytene spermatocytes and round spermatids. Therefore, an effect of hormones on the p & l secretion by Sertoli cells would mainly influence the latter cells. However, also elongated spermatids (step 19) were affected by hypophysectomy (Russell & Clermont, 1977). Further investigations of the metabolism at different stages of germ cell development are required to clarify the precise role of metabolic interactions between germ cells and Sertoli cells in the effect of hormones on spermatogenesis.

5.2. Influence of germ cells on Sertoli cells

The effect of many agents on spermatogenesis is thought to be mediated by Sertoli cells (Chapter 1). In this thesis (section 4.3.) it was shown that Sertoli cells can influence synthetic activities of pachytene spermatocytes and round spermatids. However, the relationship between Sertoli cells and germ cells is probably not a one-way influence and several observations support an effect of germ cells on Sertoli cells.

Morphological studies demonstrated in Sertoli cells intracellular and membranal specializations which are associated with certain stages of developing germ cells (review: Russell, 1980). Tubules from rats, treated with busulphan or X-irradiated *in utero*, do not contain germ cells and the tubular fluid, the composition of which is probably regulated by Sertoli cells, contains a low potassium concentration, in contrast to the high potassium concentration in tubular fluid from intact rats (Levine & Marsh, 1975; Setchell et al., 1978). Preliminary experiments demonstrated an effect of co-incubation of germ cells and Sertoli cells a glycoprotein in the membranes of Sertoli cells (Galdieri et al., 1982). All these observations suggest an influence of germ cells on activities of Sertoli cells.

Co-incubation of isolated Sertoli cells and isolated germ cells can be of use in further investigations on the interaction between these two cell types (Stefanini et al., 1980; Galdieri et al., 1981; Grootegoed et al., 1982a,b). Another method which has been used to investigate the effect of germ cells on Sertoli cells uses pieces of seminiferous tubules of definite stages of the cycle of the germinal epithelium, which can be dissected with help of a "transillumination technique" (Parvinen & Vanha-

Perttula, 1972). In these tubular fractions, containing Sertoli cells with different germ cell associations, different activities, ascribed to Sertoli cells, were recently investigated (Figure 5.2). Secretory activities of Sertoli cells appear to be maximal at stages VII and VIII of the cycle in rats (Figure 5.2A). The secretion of plasminogen activator appears to vary most clearly at the different stages, but the comparison with other activities is influenced by the expression per mg cellular protein in contrast to per mm tubule length. The apparent parallelism between the different secretory activities of Sertoli cells in different stages may indicate that the cells show a general high activity in stage VII-VIII. Recent experiments indicated that phagocytosis of residual bodies, shed by late spermatids prior to spermiation, may be one of the stimuli which increase the production of plasminogen activator by Sertoli cells at stages VII and VIII of the cycle (Lacroix et al., 1982). An increase in activities of Sertoli cells could also be caused by a high demand from the germ cells present during this stage. The carbohydrate metabolism by Sertoli cells might be stimulated to some extent by the number of germ cells, which consume the p & l secreted by Sertoli cells. In contrast to the secretory activity of Sertoli cells, the binding of FSH and the FSH-stimulated cyclic AMP production (in the presence of MIX) are minimal in stages VII-VIII (Fig. 5.2B). These studies were all performed with adult (rat) testes, because complete spermatogenesis is required to recognize the stages to be dissected. The response of Sertoli cells to FSH and the effect of FSH on spermatogenesis in intact rats decreases with age (sections 4.2.3, 5.4). This may explain why, in spite of the stage-dependent FSH binding and intracellular cAMP increase after stimulation with FSH (in the presence of MIX), only a small stage-independent increase in ABP secretion was observed after stimulation with FSH in the presence or absence of MIX (Ritzén et al., 1982). Therefore, a possible role of the stage dependency of FSH binding and cAMP response yet remains to be clarified.

Comparison of the activities of Sertoli cells incubated either in the presence of stage-specific associations of germ cells or in the presence of isolated germ cells could throw some light on the influence of germ cells on Sertoli cells.

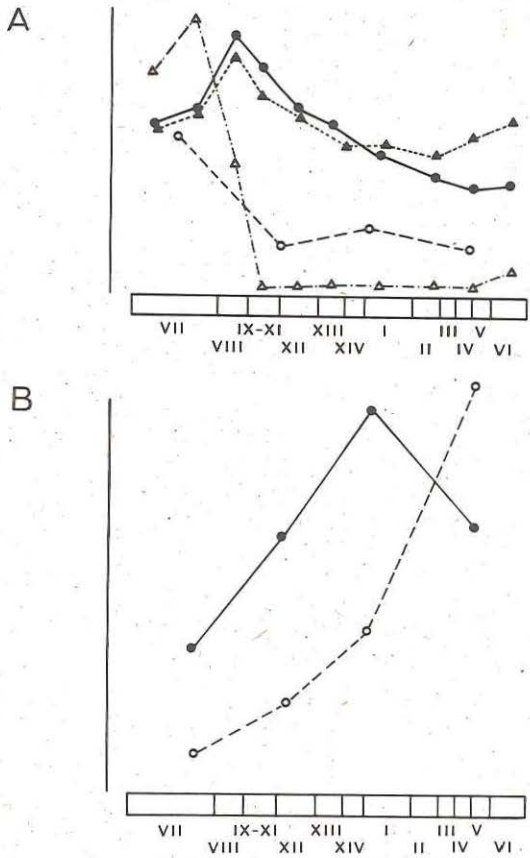


Figure 5.2. Characteristics of Sertoli cells from different stages of the spermatogenic cycle in rats.
Fragments of seminiferous tubules containing different stages of the cycle of the germinal epithelium were dissected, according to Parvinen & Vanha-Perttula (1972). In some experiments different stages were pooled.
abscissa - stages of the cycle
ordinate - relative units

A. Secretion products from Sertoli cells present in the medium after approximately 24 h of incubation:
●——● ABP (Ritzén et al., 1982)
O---O aromatase inhibitor (Boitani & Ritzén, 1981)
▲...▲ somatomedin-like activity (Johnsonbaugh et al., 1982)
Δ.-.Δ plasminogen activator (Lacroix et al., 1981)

B. ●——● binding of FSH } (Parvinen et
O---O FSH-stimulated cAMP production from 0-4 h } al., 1980)

5.3. Effects of interference with carbohydrate metabolism on germ cell development in vivo

5.3.1. Diabetes and spermatogenesis

The metabolic interrelationship of carbohydrate metabolism of germ cells and Sertoli cells as described in this thesis (section 4.3) could imply that in vivo disturbances of carbohydrate metabolism, which occur in diabetes mellitus, affect spermatogenesis directly. A review of the literature concerning the effect of diabetes on male reproduction in man gives contradictory information about the severity of the disturbances observed in different studies, mainly as a result of the variation in the severity of diabetes between the different patients (Rodriguez-Rigau, 1980). More controlled studies can be performed on laboratory animals where diabetes can be induced by injection of alloxan or streptozotocin which destroy the insulin-producing pancreatic islet tissue. In recent studies using the experimentally induced diabetes model, streptozotocin was preferred as diabetogen, because alloxan was thought to cause considerable damage unrelated to its diabetogenic action on beta islet cells (Oksanen, 1975; Paz et al., 1978a). In adult rats, the effect of streptozotocin-diabetes resulted, after at least 6 weeks, in a decreased diameter of the seminiferous tubules (Oksanen, 1975). In addition, a reduced production of spermatozoa and/or a decrease in fertility was observed (Paz et al., 1978a,b; Paz & Homonnai, 1979). However, the effect on testicular histology was not uniform in all animals. One group of rats showed a sloughing of the germinal epithelium at the early spermatocyte stage, whereas another group showed all steps of spermatogenesis, but an overall decrease of germ cell density in the epithelium (Oksanen, 1975). In rats, injected with streptozotocin before spermatogenesis was fully developed, viz. at 30 days of age, development of germ cells was completed but with a very low yield, judged by the number of spermatozoa observed in the epididymis (Paz et al., 1978a). These observations suggest a quantitative rather than a qualitative effect of streptozotocin-induced diabetes on germ cell development.

The effect of diabetes on spermatogenesis may be a direct or an indirect effect on the seminiferous tubules. The observation that insulin stimulates the secretory activity of Sertoli cells in vitro (section

4.2.3) may suggest that at least part of the effect of diabetes on spermatogenesis is exerted directly on the Sertoli cells. The increase of testicular ABP in 4 week streptozotocin-diabetic rats was thought to reflect an in vivo effect of diabetes on Sertoli cells (Murray et al., 1981). However, a concomitant decrease in epididymal ABP may indicate that not ABP secretion, but ABP transport is diminished. Indirect interference with spermatogenesis by an effect of insulin on cells outside the seminiferous tubules is supported by a decrease in FSH and testosterone levels in serum of streptozotocin-diabetic rats (Paz et al., 1978a,b; Murray et al., 1981). Direct effects of insulin or streptozotocin-diabetes on the testosterone production by Leydig cells appear small (Murray et al., 1981; Adashi et al., 1982) and therefore the reduced testosterone levels in the serum are probably caused by the decreased LH levels observed in streptozotocin-diabetic rats (Paz et al., 1978a). Possibly FSH - as well as LH secretion by the pituitary is reduced. In this respect it has been suggested that the stimulation of gonadotropin secretion by LHRH was defective in streptozotocin-diabetic rats (Paz et al., 1978a).

In summary, streptozotocin-induced diabetes appears to affect the levels of FSH and testosterone, hormones which are required for spermatogenesis to occur. When this experimentally induced diabetes is a proper model for spontaneous diabetes, these hormonal changes may play a role in the observed disturbances of spermatogenesis in diabetic males. In addition, a direct action of insulin upon Sertoli cells may influence spermatogenesis, possibly by an effect on the p & l secretion.

5.3.2. Possible relationship between the effect of some chemicals on spermatogenesis and the carbohydrate metabolism of Sertoli cells and developing germ cells

Impairment of spermatogenesis does not only result from hormonal disturbances, but can be caused by many other influences, such as the administration of some chemicals. In this section agents, which possibly affect spermatogenesis through interference with the carbohydrate metabolism of Sertoli cells and germ cells, will be considered (Fig. 5.3). Degeneration of all germ cells, except spermatogonia, was observed after feeding of the drugs 5-thio-D-glucose (Zysk et al., 1975; Homm et al., 1977; Lobl &

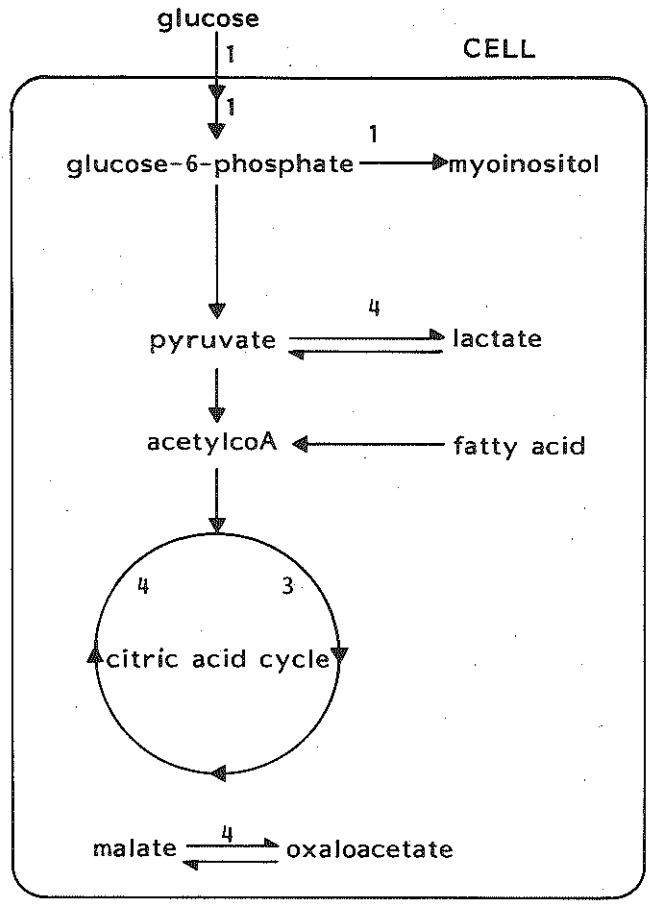


Figure 5.3. Theoretical sites of interference with carbohydrate metabolism by drugs discussed in section 4.3.

1 - 5-thio-D-glucose
2 - nitrofurans
3 - fluoroacetate
4 - gossypol

Porteus, 1978; Majumdar & Udelsman, 1979; Majumdar et al., 1979), nitrofurazones (Gomes, 1970; Hagenäs et al., 1978; Setchell, 1978), fluoroacetate or fluoroacetamide (Gomes, 1970; Setchell, 1978; Sullivan et al., 1979) and gossypol or gossypol acetic acid (National Coordinating Group on Male Antifertility Agents, China, 1979; review: Prasad & Diczfalusy, 1982). After discontinuation of the drugs spermatogenesis was restored in most cases.

5-Thio-D-glucose is an analog of D-glucose and inhibits the transport of D-glucose across plasma membranes (Whistler & Lake, 1972). However, Davies & Meanock (1981) did not observe a decrease in uptake of (^{3}H)deoxyglucose into testes of thio-glucose-treated adult mice. Thio-glucose was reported to decrease protein synthesis in isolated round and elongated spermatids to 75-55% of the controls, but the drug had no effect on spermatocytes (Nakamura & Hall, 1976, 1977). However, these experiments have been performed in the absence of pyruvate and lactate. 5-Thio-glucose-6-phosphate, which is formed inside the cell (Whistler & Lake, 1972) gave competitive inhibition with testicular myo-1-phosphate synthase, the enzyme that catalyzes myo-inositol formation from glucose-6-phosphate (Burton & Wells, 1977). Hence, 5-thio-D-glucose may influence spermatogenesis at different sites, which are interdependent. Firstly, the drug may exert a direct effect on spermatids. Although the effect in vitro appeared small, this may in vivo eventually cause degeneration of the cells. However, although spermatocytes do degenerate after treatment in vivo, these cells were not affected by the drug in vitro. Secondly, thio-glucose may interfere specifically with myo-inositol synthesis. Secretion of this polyol was shown to occur in Sertoli cells (Robinson & Fritz, 1979). However, a role of myo-inositol in spermatogenesis is still unknown and administration of a high dose of 5-thio-D-glucose for 3 days caused no decrease, but an increase of the testicular myo-inositol levels (Burton & Wells, 1977). Thirdly, inhibition of glucose uptake by Sertoli cells will reduce p & l secretion by Sertoli cells, which could cause the degeneration of pachytene spermatocytes and round spermatids observed in the testes of the thio-glucose-treated animals. The survival of Sertoli cells and spermatogonia after treatment may result from the difference in carbohydrate dependence between these cells and pachytene spermatocytes and round spermatids, which was described in sections 4.3.1 and 5.1.

It has been suggested that nitrofurans inhibit the conversion of pyruvate to acetyl-CoA (Gomes, 1970). Fluoracetamide is converted in vivo

to fluoracetate (Matsamura & O'Brien, 1963) and further to fluorocitrate, which inhibits the tricarboxylic acid cycle enzyme cis-aconitase (Peters, 1952). Hence, nitrofurans and fluoroacetate may inhibit oxidation of p & l by pachytene spermatocytes and round spermatids and in this way cause degeneration of the cells. Cells from the seminiferous tubules which are able to use glucose and/or fatty acids as energy yielding substrates (Sertoli cells and spermatogonia?) remain viable.

Gossypol can affect developing germ cells, but also epididymal spermatozoa (Prasad & Diczfalusy, 1982). Among the biochemical effects reported of gossypol, the inhibition of lactate dehydrogenase-C_4 in rodents and man and the inhibition of malate dehydrogenase in rodents appear particularly interesting (Lee & Malling, 1981). Inhibition of these enzymes may impair pyruvate and/or lactate oxidation by germ cells. In addition, the redox state of the cells may be affected, because the conversion of pyruvate to lactate, which was shown to occur in spermatocytes and spermatids (section 4.1.2), may be required to maintain a proper NAD level in the cells. In addition, impairment of transport of reducing equivalents over the mitochondrial membrane, which may be mediated by the malate-aspartate shuttle or by a lactate dehydrogenase-C_4-mediated shuttle (Storey & Kayne, 1977; Calvin & Tubbs, 1978; Blanco, 1980), would affect the redox state of germ cells.

The rapidity and severity of the reported effects of the drugs on spermatogenesis was dependent on the dose. The doses necessary to obtain an effect on spermatogenesis did not or hardly affect other tissues, and Sertoli cells and spermatogonia always remained present during treatment. The actions of these drugs *in vivo* appear to support the *in vitro* studies, which show the exceptional dependence of pachytene spermatocytes and round spermatids on p & l oxidation.

5.4. Rôles of FSH and testosterone in relation to age

Both FSH and testosterone are required for completion of spermatogenesis, but it is thought that the relative importance of these hormones changes with age. Hence, for the evaluation of the hormone effects studied *in vitro* it is important to assemble more information about the rôles of FSH and testosterone at different ages *in vivo*. The studies on this subject, which were performed mainly with rats, can be divided in two

groups, viz.: 1) the effect of hormones during initiation of spermatogenesis in the immature testis and during restoration of spermatogenesis in the regressed testis, and 2) the effect of hormones on the maintenance of spermatogenesis in the adult testis. In this section only results obtained with rats will be discussed.

Initiation and restoration of spermatogenesis

Hypophysectomy of 24-29-day-old rats (i.e. during the first wave of spermatogenesis) resulted, after 4-7 days, in a stop of spermatogenesis beyond the pachytene stage (Collins et al., 1981; section 4.3.3) and spermatocytes had disappeared after 2 months following hypophysectomy (Lostroh, 1969). Daily injection of FSH starting directly after hypophysectomy of 28 day old rats supported spermiogenesis quantitatively, judged by adenylcyclase as a marker for late spermatids, whereas testosterone only supported development to step 8-12 spermatids qualitatively (Collins et al., 1981). Either FSH or testosterone can be selectively suppressed prior to or during the first wave of spermatogenesis, by treatment with antisera against either FSH or LH from birth (Chemes et al., 1979) or from 20 days of age (Madhwa Raj et al., 1976), or by treatment with a gonadotropin-suppressing dose of testosterone propionate from birth (Steinberger & Ducket, 1965). Under such conditions spermatogenesis proceeded at least up to and including pachytene spermatocytes, but the yield of spermatocytes was below the yield of intact controls (Madhwa Raj et al., 1976; Chemes et al., 1979). Some round spermatids and occasionally some elongated spermatids (step 15) were observed (Steinberger & Duckett, 1965). It must be kept in mind, however, that suppression of FSH or testosterone levels in these experiments may have been incomplete.

When both FSH and testosterone had been absent for 1-6 months after hypophysectomy of immature (28-42 day old) or adult rats, complete or partial regression of spermatogenesis occurred. In these rats histology, progression of (^{3}H)thymidine (which is incorporated in spermatogonia and preleptotene spermatocytes) or testicular levels of carnitine acetyl transferase (as a marker for pachytene spermatocytes) indicated that FSH -, LH - or testosterone treatment supported development of pachytene spermatocytes qualitatively, and no or only very few spermatids and spermatozoa were present (Lostroh, 1963a,b, 1969; Go et al., 1971). In

one study a similar effect of FSH, but no effect of LH was observed (Sivelle et al., 1978). In these regressed testes, FSH and LH or testosterone together improved the yield of all germ cells and at least the number of pachytene spermatocytes was restored to the normal level (Lostroh, 1969; Go et al., 1971).

In summary, the presence of either FSH or testosterone appeared to support initiation and restoration of spermatogenesis qualitatively up to and including formation of pachytene spermatocytes. When both FSH and testosterone were present during development of the first wave of pachytene spermatocytes, spermiogenesis appeared to be supported by FSH, but not by testosterone. Quantitative restoration of spermatogenesis is supported most effectively by FSH and LH or testosterone together.

Maintenance of spermatogenesis

For adult rats it is generally accepted that testosterone is required to maintain complete spermatogenesis qualitatively (review: Steinberger, 1971). However, two aspects of hormonal regulation of adult spermatogenesis are still a matter of debate, viz.: does testosterone alone maintain spermatogenesis quantitatively and what is the possible role of FSH in adult rats? Since the review by Steinberger (1971) additional quantitative studies have been performed. Quantitative maintenance of spermatogenesis was observed when testosterone propionate or dihydrotestosterone injections were started on the day of hypophysectomy or the following day (Matsuyama et al., 1971; Ahmad et al., 1975). However, other investigators observed no complete quantitative maintenance of spermatogenesis when injections of testosterone propionate, dihydrotestosterone or LH were started within 24 h after hypophysectomy (Russell & Clermont, 1977; Chowdhury, 1979) or 2-5 days after hypophysectomy (Clermont & Harvey, 1967; Elkington & Blackshaw, 1974; Chowdhury & Tcholakian, 1979). Administration of FSH and LH or testosterone propionate together appeared to maintain spermatogenesis quantitatively when treatment started 12 h after hypophysectomy (Russell & Clermont, 1977), but not when hormones were injected starting 2 days after hypophysectomy (Elkington & Blackshaw, 1974).

In summary, most studies indicate that testosterone alone cannot completely maintain spermatogenesis quantitatively and it appears likely that FSH is required in adult rats to supplement the effect of testosterone.

Conclusion

Both FSH and testosterone appear to influence development of germ cells *in vivo* in immature as well as in adult rats. However, *in vitro* only FSH stimulated the p & 1 secretion by Sertoli cells from immature rats, whereas testosterone did not (section 4.2.3). These observations may reflect that under the present *in vitro* conditions testosterone cannot stimulate Sertoli cells, or that the mechanism of action of testosterone on spermatogenesis does not involve an effect of testosterone on the carbohydrate metabolism of Sertoli cells.

5.5. Regulation of spermatogenesis

The literature summarized in the previous section indicated that in the absence of hormones few germ cells develop, whereas in the presence of FSH and/or testosterone a larger or maximal number of germ cells go through spermatogenesis. No effect of hormones on the duration of the spermatogenic cycle was observed (Clermont & Harvey, 1965). This effect of hormones on germ cell numbers rather than on germ cell differentiation was confirmed by measurements of synthetic activities in pachytene spermatocytes. After hypophysectomy the number of cells which were inactive in RNA synthesis was increased, whereas the pattern of qualitative RNA synthesis did not change (Grootegoed et al., 1979). Such a quantitative regulation of the synthetic activities and survival of germ cells may reflect an effect of hormones upon the supply of energy yielding substrates to the cells. If the amount of energy-rich substrates is limiting in the micro milieu around the germ cells, an increase of the substrate supply by stimulation of Sertoli cells may influence the number of developing germ cells. In addition to hormones, many other agents or conditions have an effect on the number of germ cells *in vivo*, such as some drugs (section 5.4.2), diabetes (section 5.4.1) and heat (Setchell, 1978).

In vitro, isolated pachytene spermatocytes or round spermatids maintain a specific pattern of RNA - and protein synthesis or glycoprotein fucosylation for 12, 24 and 20 h respectively when incubated in the presence of p & 1 (Grootegoed et al., 1977a; 1982c; section 3.2), whereas they are virtually inactive and degenerate within 24 h in the absence of p & 1 (section 4.1.1). Because Sertoli cells are capable to supply p & 1 to germ cells (section 4.2.1), these observations

imply that the presence of Sertoli cells, at least during short-term incubations, is primarily important for the energy supply, via p & l , to pachytene spermatocytes. This appears to be confirmed by the reduction in the number of germ cells in seminiferous tubules in vitro after p & l depletion by removal of glucose from the medium (section 4.3.1). As the p & l secretion by Sertoli cells can be stimulated by FSH in vitro (section 4.2.3), it can be suggested that p & l may be involved in the quantitative regulation of spermatogenesis by hormones in vivo. Attempts to induce such an FSH effect on germ cells in vitro have failed, probably because the incubation conditions in vitro in the absence of hormones are more favourable for survival of pachytene spermatocytes and round spermatids than the conditions in vivo after hypophysectomy (section 4.3.3).

In addition to p & l , other factors from Sertoli cells probably affect the activities of germ cells. This is most clearly suggested by the observation that isolated pachytene spermatocytes and round spermatids incubated in the presence of p & l start degenerating after maximally 48 h, whereas these cells survive for at least 4 days in isolated fragments of seminiferous tubules (section 4.3.1). Yet, p & l are the most important secretion products from Sertoli cells, which maintain activities of pachytene spermatocytes and round spermatids during short-term incubations (section 4.3.2). Under conditions that energy supply to germ cells is not limiting, other factors from Sertoli cells which are essential for germ cell development may exert an effect.

In conclusion, the quantitative nature of the regulation of spermatogenesis in vivo by hormones and some other agents suggests, that energy supply to germ cells may be involved. The FSH-stimulated secretion of p & l by Sertoli cells and the effect of these substrates on pachytene spermatocytes and round spermatids in vitro indicate that p & l may be involved in the effect of hormones on spermatogenesis.

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SUMMARY

Development of male germ cells in mammalian testes takes place within seminiferous tubules in the close proximity of Sertoli cells. Initiation of spermatogenesis in immature rats and maintenance of spermatogenesis in adult animals requires the presence of FSH and testosterone, and it is generally thought that the effect of these hormones on developing germ cells is mediated by Sertoli cells. Yet, the biochemical background of the action of Sertoli cells upon germ cells is far from clear. The aim of the experiments presented in this thesis was to contribute to the elucidation of the interaction between Sertoli cells and germ cells, in particular between Sertoli cells and pachytene spermatocytes and round spermatids, two stages of germ cell development which are sensitive to the presence of hormones in vivo. Some aspects of spermatogenesis and the scope of this thesis have been described in Chapter 1.

Chapter 2 gives a short summary of the materials and methods used to analyze activities of germ cells and Sertoli cells.

Experiments described in Chapter 3 and Appendix Paper I indicate that pachytene spermatocytes do not require the presence of Sertoli cells to maintain a cell-specific pattern of protein synthesis (analyzed with two-dimensional electrophoresis) during incubation for at least 24 hours.

The dependence of testicular tissue on glucose as a substrate has been recognized for some time and it was concluded from experiments with testicular tissue that pachytene spermatocytes and round spermatids are very dependent on glucose. Experiments described in Chapter 4, section 1 and Appendix Paper II indicate that after isolation of these germ cells, glucose could not maintain synthetic activities in pachytene spermatocytes and round spermatids, whereas the catabolites pyruvate and lactate were more effective in this respect. When incubated in a chemically defined medium in the absence of pyruvate and lactate, isolated pachytene spermatocytes degenerated within 12 hours, whereas these cells survived for at least 24 hours during incubation in the presence of pyruvate and lactate. It was shown (Chapter 4, section 2, Appendix Papers III, IV) that Sertoli cells can secrete pyruvate and lactate, and that the rate of secretion can be changed by the concentration of exogenous glucose, the age of the rats from which testes were obtained and by the hormones FSH and insulin. Changes in the concentration of exogenous glucose also influenced synthetic activities

and/or survival of pachytene spermatocytes and round spermatids when the cells were incubated in the presence of Sertoli cells (Chapter 4, section 3, Appendix Paper III). Correlation of the effect of spent medium of Sertoli cells on synthetic activities of isolated germ cells, and the amount of pyruvate and lactate in this medium, suggests that pyruvate and lactate are major secretion products from Sertoli cells which maintain synthetic activities in germ cells during short-term incubations (Chapter 4, section 3, Appendix Paper IV). The present results suggest that Sertoli cells can influence activities and survival of pachytene spermatocytes and round spermatids by supply of pyruvate and lactate to the cells.

Experiments described in Chapter 4, section 3 indicate that the survival of pachytene spermatocytes in seminiferous tubules incubated in the absence of hormones is virtually optimal, in contrast to the poor survival of these cells in testes of hypophysectomized rats. Therefore, no effect of FSH on the survival of pachytene spermatocytes could be observed in vitro under the incubation conditions used. The amount of substrates present in vitro may be enough for unstimulated Sertoli cells to produce sufficient pyruvate and lactate for the energy supply of these germ cells.

In conclusion, the presence of Sertoli cells appears to be required primarily to maintain the overall rate of synthetic activities in pachytene spermatocytes and round spermatids, rather than to support synthesis of specific proteins in germ cells. Regulation of the yield of germ cell development is an important aspect of hormonal regulation of spermatogenesis. From the results in Chapter 4 it is suggested that secretion of pyruvate and lactate by Sertoli cells is involved in hormonal regulation of the number of developing germ cells.

SAMENVATTING

De ontwikkeling van mannelijke zaadcellen in de zaadballen van zoogdieren vindt plaats binnen zaaddragende buisjes (de tubuli seminiferi) in nauw kontakt met Sertoli-cellen. Voor de aanzet tot de ontwikkeling van zaadcellen in de jonge rat en het in stand houden van de zaadcelontwikkeling in de volwassen rat, is de aanwezigheid van de hormonen follitropine (FSH) en testosteron noodzakelijk. Er zijn diverse aanwijzingen dat de Sertoli-cellen een belangrijke rol spelen bij het effekt van hormonen op de zich ontwikkelende zaadcellen. De biochemische achtergrond van de invloed van Sertoli-cellen op zich ontwikkelende zaadcellen is echter nog niet duidelijk. Via de in dit proefschrift beschreven experimenten is geprobeerd een beter inzicht te krijgen in de mogelijke interaktie tussen Sertoli-cellen en zaadcellen, en wel die tussen Sertoli-cellen en pachyteenspermatocyten en ronde spermatiden, twee stadia uit de ontwikkeling van zaadcellen die gevoelig zijn voor de aanwezigheid van hormonen in het levende dier.

De ontwikkeling van zaadcellen en de regulatie hiervan door hormonen is in het kort beschreven in Hoofdstuk 1, evenals het doel van dit proefschrift.

In Hoofdstuk 2 is een kort overzicht gegeven van de materialen en methodes die gebruikt zijn om enkele aktiviteiten van zaadcellen en Sertoli-cellen te onderzoeken.

De in Hoofdstuk 3 en Aanhangsel I beschreven experimenten wijzen erop, dat pachytene spermatocyten, in afwezigheid van Sertoli-cellen, gedurende minstens 24 uur een aantal eiwitten kunnen synthetiseren die specifiek zijn voor deze cellen.

Het was al bekend dat het weefsel in de zaadbal afhankelijk is van suikers, zoals glucose, als bron van energie, en uit experimenten met de gehele zaadbal was gekonkludeerd, dat pachyteenspermatocyten en ronde spermatiden glucose nodig hebben. De in Hoofdstuk 4, deel 1 en Aanhangsel II beschreven experimenten wijzen erop, dat na isolatie van deze celtypes, de opbouw van eiwitten en ribonucleïnezuren in pachyteenspermatocyten en ronde spermatiden niet in stand gehouden werd in aanwezigheid van glucose. De afbraakprodukten van glucose, pyruvaat en lactaat daarentegen waren effektiever in dit opzicht. Tijdens kweek zonder pyruvaat en lactaat beginnen pachyteenspermatocyten binnen 12 uur te degenereren,

terwijl de cellen minstens 24 uur in goede konditie lijken te zijn wanneer ze gekweekt worden in aanwezigheid van pyruvaat en lactaat. Het is aangetoond (Hoofdstuk 4, deel 2 en Aanhangsels III, IV), dat Sertoli-cellen pyruvaat en lactaat kunnen uitscheiden en dat de hoeveelheid die uitgescheiden wordt, kan veranderen onder invloed van de koncentratie glucose, de leeftijd van de ratten waaruit de zaadballen werden verkregen, en de hormonen follitropine en insuline. Verandering van de koncentratie glucose buiten de cellen beïnvloedt ook de opbouw van ribonucleïnezuren en/of de overleving van pachyteenspermatocyten en ronde spermatiden wanneer deze cellen gekweekt worden in aanwezigheid van Sertoli-cellen (Hoofdstuk 4, deel 3 en Aanhangsel III). Vergelijking van het effekt van gebruikt kweekmedium van Sertoli-cellen op de aanmaak van eiwitten in geïsoleerde spermatocyten en spermatiden met het effekt van de hoeveelheid pyruvaat en lactaat in dit medium, suggereert dat pyruvaat en lactaat relatief de belangrijkste uitscheidingsprodukten van Sertoli-cellen zijn, welke, op korte termijn, de synthetische aktiviteiten in deze zaadcellen kunnen handhaven (Hoofdstuk 4, deel 3 en Aanhangsel IV). De verkregen resultaten suggereren, dat Sertoli cellen een invloed kunnen hebben op aktiviteiten en overleving van pachyteenspermatocyten en ronde spermatiden door deze cellen te voorzien van pyruvaat en lactaat. De in Hoofdstuk 4, deel 3 beschreven experimenten wijzen erop, dat in zaaddragende buisjes, gekweekt zonder toevoeging van hormonen, de overleving van pachyteenspermatocyten bijna optimaal is, dit in tegenstelling tot de sterfte van deze cellen in zaadballen van ratten zonder hypofyse. Onder deze kweekomstandigheden kon geen effekt van FSH op de overleving van spermatocyten worden waargenomen. Mogelijk zijn er in kweek zoveel substraten aanwezig, dat ongestimuleerde Sertoli-cellen voldoende pyruvaat en lactaat kunnen produceren voor de energievoorziening van deze zaadcellen.

Op grond van de beschreven resultaten is gekonkludeerd, dat de aanwezigheid van Sertoli cellen belangrijker is om aktiviteiten van pachyteenspermatocyten en ronde spermatiden te handhaven dan om de aanmaak van bepaalde eiwitten te bevorderen. Regulatie van het aantal zich ontwikkelende zaadcellen is een belangrijk effekt van hormonale regulatie van de spermatogenese. De gevonden resultaten tonen aan, dat uitscheiding van pyruvaat en lactaat door Sertoli cellen mogelijk betrokken is bij de hormonale regulatie van de overleving van zich ontwikkelende zaadcellen.

LIST OF NON-STANDARD ABBREVIATIONS

ABP	- androgen binding protein
bt_2cAMP	- dibutyryl cyclic AMP; N^6-2'-O-dibutyryl-adenosine-3':5'-monophosphate
1C	- amount of DNA present in a haploid gamete
1D	- one-dimensional
2D	- two-dimensional
ED_{50}	- dose required for half-maximal stimulation
FSH	- follicle-stimulating hormone (follitropin)
LDH	- lactate dehydrogenase
LH	- luteinizing hormone (lutropin)
MIX	- 3-isobutyl-1-methylxanthine
p & l	- pyruvate and lactate
S1	- unit for characterization of FSH preparations = activity in one mg of ovine NIH-FSH-S1

LIST OF TRIVIAL NAMES

Trivial name used in this thesis	Systematic name
alloxan	- 2,4,5,6(1H,3H)-pyrimidinetetrone
gossypol	- 1,1',6,6',7,7'-hexahydroxy-3,3'-dimethyl, 5,5'-bis-isopropyl[2,2'-bi-naphtalene]-8,8'-dicarboxaldehyde
nitrofurazone	- 5-nitro-2-furaldehyde-semicarbazone
oestradiol	- 1,3,5(10)-oestratriene-3,17β-diol
streptozotocin	- 2,deoxy-2[(methylnitrosoamino)-carbonyl]-amino]-glucopyranose
testosterone	- 17β-hydroxy-4-androsten-3-one
thioglucose	- 5-thio-α-D-glucopyranose

NAWOORD

Graag wil ik iedereen bedanken die bijgedragen heeft aan het ontstaan van dit proefschrift. Met name:

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Nicolet Jutte.

CURRICULUM VITAE

Ik ben geboren in Schiedam op 19 december 1952 maar getogen in Rotterdam, waar ik in 1970 het diploma gymnasium-β behaalde op lyceum "Maria Virgo". In hetzelfde jaar begon ik met de biologiestudie aan de Rijksuniversiteit Leiden. Na het kandidaatsexamen in december 1973, met als tweede hoofdvak scheikunde (B4), behaalde ik in december 1977 het doktoraaldiploma, met de vakken chemische endocrinologie, celbiologie en dierfysiologie. Tijdens de postkandidaatsfase assisteerde ik verschillende malen bij praktika in mikrotechniek en histologie. Via FUNGO ben ik vanaf maart 1978 tot en met december 1981 werkzaam geweest op de afdeling Biochemie II (Chemische Endocrinologie) van de Erasmus Universiteit Rotterdam.

Appendix Paper I

(submitted to Int. J. Androl.)

MAINTENANCE OF THE PATTERN OF PROTEIN SYNTHESIS IN ISOLATED PACHYTENE SPERMATOCYTES

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SUMMARY

Protein synthesis in isolated pachytene spermatocytes and round spermatids was investigated by means of one- and two-dimensional polyacrylamide gel electrophoresis. The patterns of newly-synthesized proteins of spermatocytes and spermatids showed, next to many proteins common to both spermatogenic cell types, several cell specific proteins. Electrophoretic patterns of radioactively labelled proteins of pachytene spermatocytes were virtually the same after incorporation of (^{35}S) methionine from 0-2 h or from 24-26 h after isolation. In contrast, one-dimensional electrophoretic analysis of proteins newly-synthesized by Sertoli cells showed marked changes of the pattern within 26 h of incubation.
It can be concluded that, although Sertoli cells may support the rate of protein synthesis in developing germ cells, the presence of Sertoli cells is not required for the maintenance of a specific pattern of protein synthesis by spermatocytes during incubation for at least 26 h.

INTRODUCTION

Close association of developing germ cells and active Sertoli cells in the germinal epithelium is thought to be essential for spermatogenesis. This is supported by morphological evidence showing the contact between Sertoli cells and germ cells, which is extended by cytoplasmic and membranal specializations on the boundary of the two cell types (review:

Russell, 1980). Moreover, the greater part of spermatogenesis takes place within a tubular compartment which is separated from the interstitium by Sertoli cells. The regulation of spermatogenesis by FSH and testosterone is thought to be mediated by Sertoli cells (Fritz, 1978). Mid-pachytene spermatocytes and round and elongated spermatids may be dependent on Sertoli cell activities, in particular at stages VII-VIII of the cycle of the seminiferous epithelium in rats, because at these stages germ cells rapidly degenerate after hypophysectomy (Russell & Clermont, 1977). It is largely unknown which biochemical activities of germ cells are primarily dependent on activities of Sertoli cells. Moreover, it is still uncertain whether Sertoli cells direct the development of germ cells, and/or support processes which are completely programmed within the germ cells.
Cellular protein synthesis can be studied in detail by qualitative analysis of proteins using two-dimensional polyacrylamide electrophoresis. With this method we investigated protein synthesis of isolated pachytene spermatocytes under conditions where support by Sertoli cells was absent.

MATERIAL AND METHODS

Preparations enriched with pachytene spermatocytes or round spermatids were obtained by fractionation of a testicular cell suspension, isolated from testes of immature rats (age 30-35 days, Wistar, substrain R-Amsterdam), by sedimentation at unit gravity (Jutte et al., 1982). These fractions were further purified on Percoll gradients by the following modification of the method of Meistrich et al., (1981). Percoll gradients were formed by centrifugation of 10 ml 30% Percoll (w/v) (Pharmacia) in calcium-free phosphate-buffered saline (PBS), containing 1 mM $MgCl_2$, 100 µg DNA ase/ml (DN25, Sigma), 2 mM sodium pyruvate and 6 mM sodium DL-lactate, during 20 min at $4^{o}C$ at 12000 g in an angle rotor (SS 34) in a Sorvall RC-2B centrifuge. The cells collected from the sedimentation column were washed twice in calcium- and magnesium-free PBS,

containing pyruvate and lactate and once in calcium- and magnesium-free PBS, containing pyruvate, lactate, 20% Percoll and 20 µg DNA ase/ml. Maximally 10^8 pachytene spermatocytes or 4.10^7 round spermatids were suspended in 1 ml of the last washing fluid, layered upon the Percoll gradients and centrifugated for 10 min at 650 g at 4°C. The cell bands which had been formed were collected with a syringe and diluted 1:3 (v/v) with PBS. Subsequently the cells were collected by centrifugation for 7.5 min at 150 g at room temperature. The preparations contained the following cells: A) mid-late pachytene spermatocytes (95.6 ± 1.5%), early primary spermatocytes (1.0 ± 0.9%), round spermatids (0.7 ± 0.6%), other types of germ cells and unidentified cells (2.4 ± 0.9%) and somatic cells (0.2 ± 0.2%) (mean ± S.D., n = 3); B) round spermatids (88.0 ± 0.9%), early primary spermatocytes (7.6 ± 2.5%), other types of germ cells and unidentified cells (4.2 ± 2.1%) and somatic cells (0.2 ± 0.3 %) (mean ± range, n = 2).

Sertoli cell aggregates (tubular fragments from sterile rats) were isolated as described previously (Jutte et al., 1982) from immature (28-day-old) rats, which had been irradiated *in utero* at day 19 of gestation (Beaumont, 1960). Following isolation, the cells were washed thrice in the medium used for incubation.

Incubation conditions

Freshly isolated pachytene spermatocytes (0.5 x 10^6 cells), round spermatids (10^6 cells) or Sertoli cells (± 200 µg protein) were incubated for 2 h in 200 µl incubation medium containing 2 mM sodium pyruvate and 6 mM DL-lactate, in the presence of 10 µCi (^{35}S)methionine. Medium without unlabelled methionine was used to obtain a high specific radioactivity of (^{35}S)methionine (final specific radioactivity 200 Ci/mmol). The incubations were performed at 32°C under a humidified atmosphere of 5% CO_2 in air in plastic tubes (10 ml) in a shaking water bath (120 cycles/min). The incorporation of (^{35}S)methionine into germ cells was linear for at least 3 h.

Spermatocytes which had been incubated in Petri dishes (32°C; 5% CO_2 in air) for 24 h in 2 ml of incubation medium containing methionine (0.6 mM) were collected by centrifugation and after three washes in incubation medium without methionine the cells were incubated with (^{35}S)methionine as described above.
After 24 h of incubation (± 200 µg protein/2 ml; 32°C; 5% CO_2 in air) Sertoli cells were attached to the bottom of plastic Petri dishes. The attached cells were washed thrice and incubated in 600 µl incubation medium, containing 30 µCi (^{35}S)methionine (final specific radioactivity 200 Ci/mmol). After incubation the Sertoli cells were detached from the plastic surface with a rubber policeman. All incubations with (^{35}S)methionine were terminated by addition of 2 volumes of 0.9% (w/v) NaCl (4°C), containing 7 mM methionine. The cells were collected by centrifugation for 10 min at 1500 g at 4°C and subsequently frozen at -80°C and stored at -20°C.

Electrophoresis

For one-dimensional (1D) electrophoresis (^{35}S)methionine-labelled cells were suspended in buffer (approximately 100 µg of protein/50 µl), containing sodium dodecyl sulphate and 2-mercapto-ethanol (Laemmli, 1970), and heated for 5 min at 100°C. The solubilized proteins were separated by electrophoresis (± 10 µg/slot) as described previously (Grootegoed et al., 1982b).
For two-dimensional (2D) electrophoresis (^{35}S)methionine-labelled whole cells were suspended in buffer (approximately 100 µg of protein/30 µl), containing Non-idet P40, ureuam and 2-mercapto-ethanol (O'Farrell, 1975) and were frozen and thawn three times. Iso-electric focussing gels with a diameter of 0.2 mm and a length of 10 cm containing 2% Ampholines (w/v) (LKB; ratio pH-range 5-7 to pH-range 3-10 4 to 1) were prepared. After pre-electrophoresis of the gels, the solubilized proteins were applied and were separated in the buffer system, as described by O'Farrell (1975). Electrophoresis was carried out under continuous cooling at 20°C at a fixed voltage of 400 V

for 12-16 h, followed by 1 h at 800 V.
After the iso-electric focussing, the gels were equilibrated during 2 h in buffer containing SDS and 2-mercapto-ethanol (Laemmli, 1970). Subsequently, the buffer was renewed and the gels were quickly frozen at -80°C and stored at -20°C. The iso-electric focussing gels were loaded onto slab gels in a solution of 1% (w/v) agarose in buffer containing SDS and 2-mercapto-ethanol (O'Farrell, 1975). The stacking gel, separating gel and the conditions of electrophoresis used were as described for one-dimensional electrophoresis (Grootegoed et al., 1982b).
After electrophoresis, the separated proteins were fixed in the slab gels (1D and 2D) with 10% (w/v) trichloro-acetic acid. For visualization of labelled proteins after separation in two dimensions, the gels were treated with 2,5-diphenyl-oxazole (PPO), to perform fluorography (Bonner & Laskey, 1974). Proteins separated in one dimension only, were made visible by using autoradiography. Unlabelled proteins were detected by staining of the proteins with PAGE Blue 23 (BDH) or with a silver staining method (Sammons et al., 1981). The slab gels were dried under vacuum and heat in a Bio-Rad model 224 gel slab dryer and subsequently exposed to Kodak XR-1 X-Omat film (Eastman Kodak Company, Rochester, New York) at -80°C.

RESULTS

Isolated pachytene spermatocytes were incubated in the presence of (^{35}S)methionine either from 0-2 h after completion of the isolation procedure or from 24-26 h after isolation. Cellular proteins within a molecular weight range of approximately 20,000-120,000 were separated with SDS-electrophoresis and analyzed with radioautography. The pattern of proteins newly-synthesized after preincubation of the isolated cells for 24 h at 32°C in chemically defined medium, was virtually identical to the pattern of proteins newly-synthesized immediately after isolation (Fig. 1). In contrast, quantitative differences were observed when the pattern of proteins newly-synthesized by freshly isolated Sertoli cells was compared with the pattern

of proteins of Sertoli cells labelled from 24-26 h after isolation. At least 8 protein bands from Sertoli cells showed more incorporation of radioactivity after labelling from 0-2 h, and at least 3 protein bands were more intensely labelled from 24-26 h. At 24 h after isolation, the morphology (examined by phase-contrast microscopy) of more than 90% of the spermatocytes was virtually indistinguishable from that of freshly isolated cells. Moreover, the incorporation of (^{3}H)leucine into acid precipitable material from spermatocytes (expressed per number of cells) was comparable when measured during 0-2 h or 24-26 h after isolation. At 48 h after isolation, however, most spermatocytes showed morphological signs of degeneration. Sertoli cells can be maintained in incubation for more than one week (Grootegoed et al., 1978).

Only a small number of proteins can be analyzed with one-dimensional electrophoresis, and the effect of a 24 h incubation period on protein synthesis in isolated spermatocytes was therefore also studied by using two-dimensional electrophoresis. Proteins with iso-electric points between 4.5 and 7.0 and molecular weights between 20,000 and 120,000 were analyzed with the gel system used. Approximately 300-400 spots could be observed after staining of the separated proteins with a very sensitive silver-stain technique, or in fluorograms of proteins labelled with (^{35}S)methionine. Fluorograms of radioactively labelled proteins, newly-synthesized by pachytene spermatocytes or round spermatids during 2 h, are shown in Fig. 2. Also with this powerful method virtually no effect of preincunation for 24 h was observed on the pattern of protein synthesis by pachytene spermatocytes (Figs. 2a and 2b). However, the protein synthesis patterns of pachytene spermatocytes and round spermatids were markedly different (Figs. 2a and 2c). Several differences between the patterns of proteins, newly-synthesized by pachytene spermatocytes and by round spermatids are indicated in the figure. At least 8 spots represent proteins which were synthesized at a much higher rate in spermatocytes than in spermatids, and at least 10 spots represent proteins which were synthesized preferentially in spermatids. These results, observed in at least three different experiments,

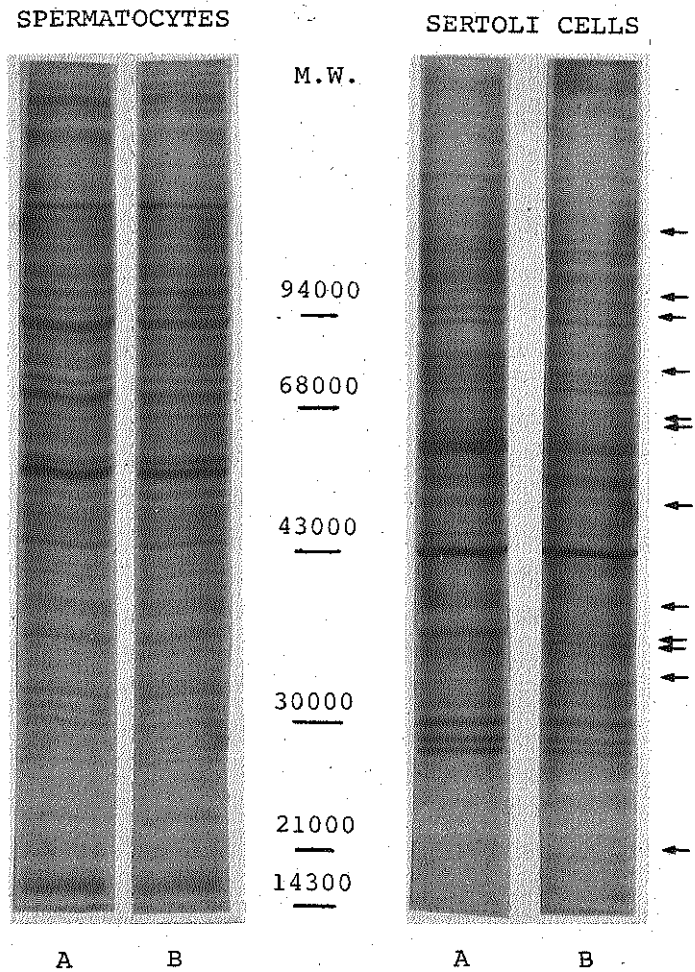
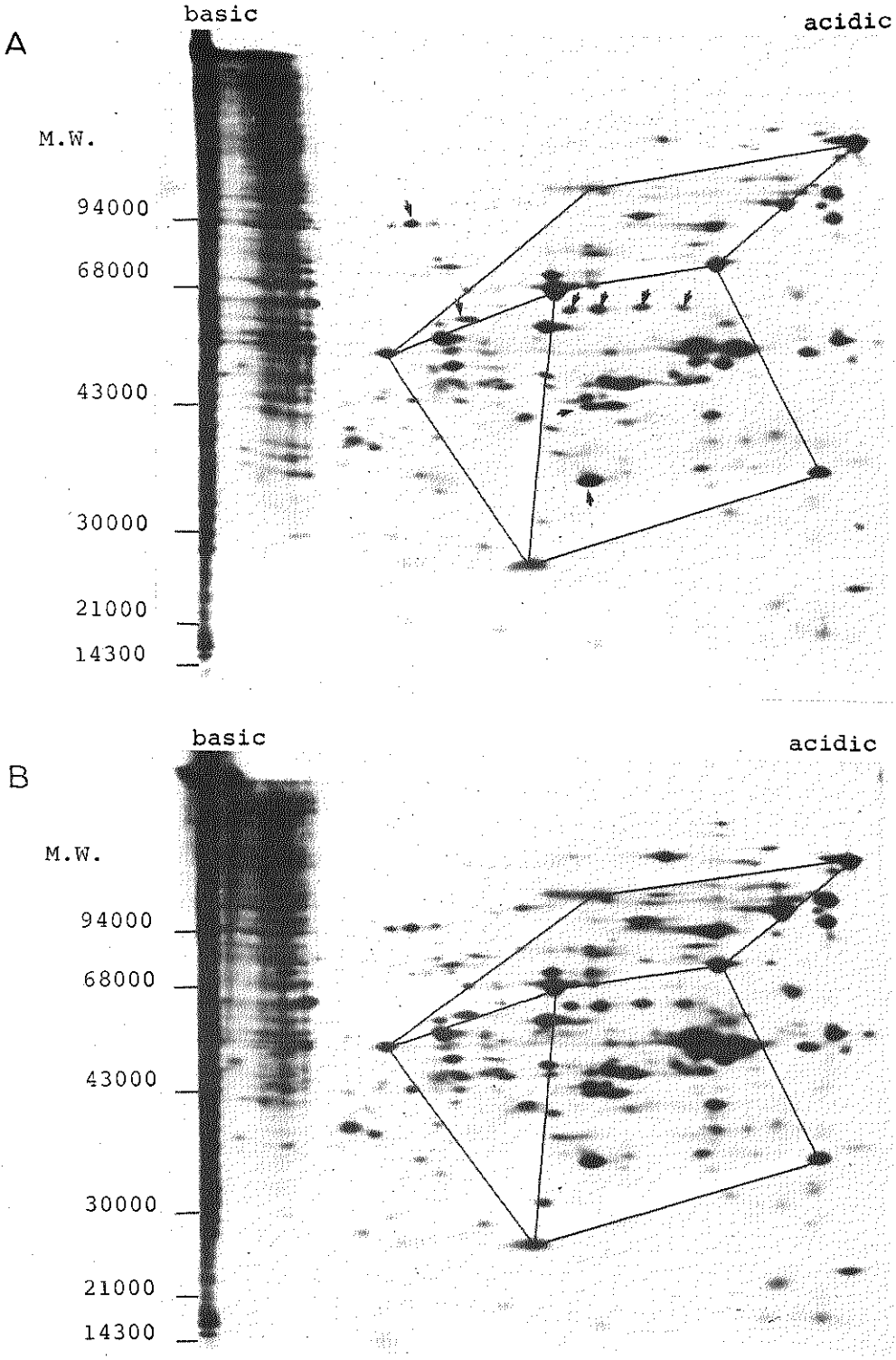


Figure 1. Radioautographic patterns obtained after one-dimensional electrophoretic analysis of newly-synthesized proteins from isolated pachytene spermatocytes or Sertoli cells.
Cells were incubated for 2 h in the presence of (^{35}S)-methionine (200 Ci/mmol) immediately after isolation (A) or after 24 h of incubation (B). Arrows indicate differences between the Sertoli cell protein bands in lanes A and B.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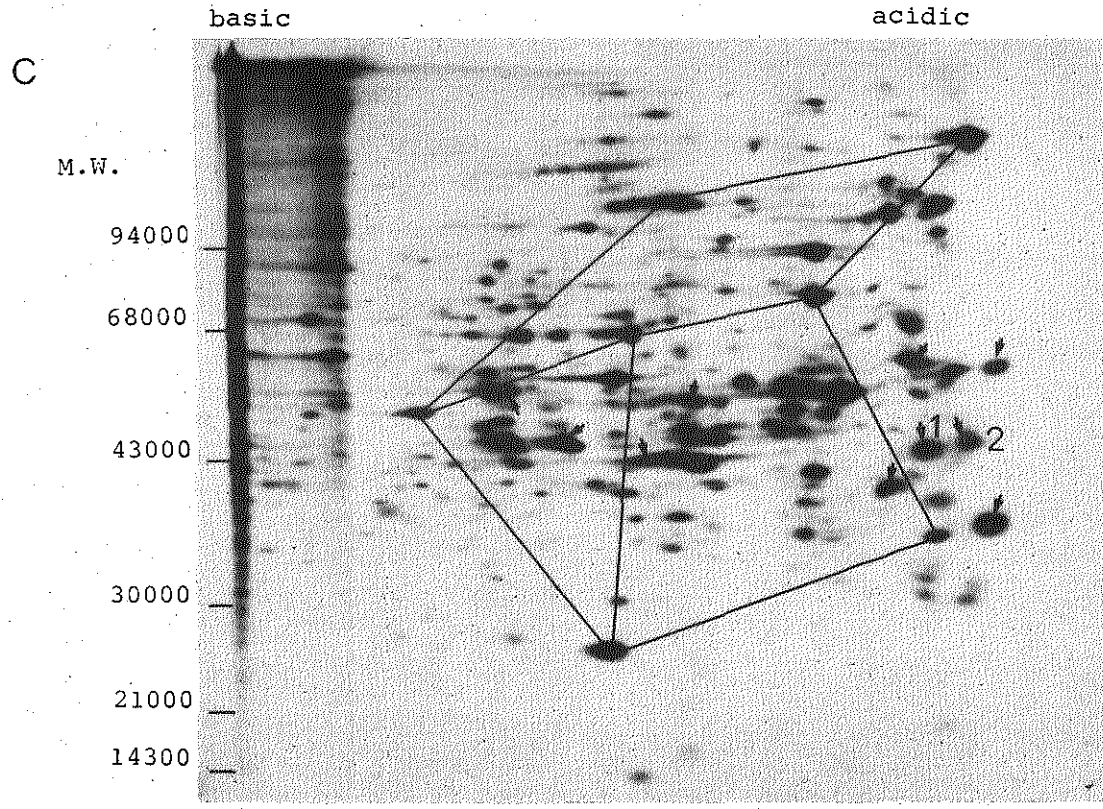

Figure 2. Fluorographic patterns obtained after two-dimensional electrophoretic analysis of newly-synthesized proteins from:
freshly isolated pachytene spermatocytes (A), pachytene spermatocytes after 24 h of incubation (B), or freshly isolated round spermatids (C). Cells were incubated for 2 h in the presence of (^{35}S)methionine. Reference spots which were present in both cell types were interconnected to compose a frame for mutual comparison of the fluorograms. Arrows indicate qualitative differences between the two cell types. Two very dense spots, which are exclusively present in spermatids, are numbered.

indicate that a specific pattern of proteins is synthesized in spermatocytes as compared to spermatids. Moreover, the pattern of protein synthesis in pachytene spermatocytes was maintained during incubation for 24 h in the absence of Sertoli cells.

DISCUSSION

The present results show marked differences between the patterns of proteins, newly-synthesized by pachytene spermatocytes or by round spermatids. We have tried to purify the germ cell preparations extensively by subjecting preparations of pachytene spermatocytes and round spermatids, collected from the sedimentation column, to a subsequent purification on Percoll gradients. A similar procedure was first described by Meistrich et al. (1981) and resulted in a significant improvement of purity. Despite this extensive purification, cross-contamination of the preparations of spermatocytes and spermatids does not allow definite conclusions with respect to absolute qualitative differences between the protein patterns of spermatocytes and spermatids. However, some very dense spots (marked 1 and 2 in Fig. 2c) present in the pattern of spermatids, are completely absent in the pattern of spermatocytes, indicating that contaminating spermatids do not contribute to the pattern of protein synthesis of spermatocytes. Changes in the patterns of protein - or RNA synthesis, concomitant with development from pachytene spermatocytes to round spermatids, have also been shown by other investigators (Grootegoed et al., 1977; Boitani et al., 1980; Kramer & Erickson, 1982). The occurrence and timing of biochemical changes during development may represent a series of events programmed within germ cells, and the execution of this program may be supported and/or directed by Sertoli cells. A change in the pattern of newly synthesized proteins, resulting from development of pachytene spermatocytes during 24 hours of incubation, is unlikely to be observed. The reason for this is that development through the pachytene spermatocyte stage takes about 13 days in rats, and the preparations of mid-late pachytene spermatocytes used in our experiments may

contain cells which differ up to 8-10 days in development. The present results show that in Sertoli cells the pattern of newly synthesized proteins was changed after 24 h of incubation. Previously, it has been shown that after isolation and incubation of Sertoli cells changes in steroid and protein secretion occur (Louis & Fritz, 1977; Grootegoed et al., 1978; Rommerts et al., 1980; Skinner & Griswold, 1982). Hence, activities of Sertoli cells can be influenced, and these changes are not related to cell death, because Sertoli cells can be maintained in incubation for more than one week. In contrast, the present results show that the pattern of proteins newly-synthesized by pachytene spermatocytes did not change after incubation of the cells for 24 h, although many cells die during a subsequent incubation period. Previously, it was reported that isolation of mouse pachytene spermatocytes and spermatids from seminiferous tubules did not result in an immediate change of the protein synthesis pattern (Boitani et al., 1980). Moreover, the electrophoretic pattern of glycoprotein fucosylation by isolated round spermatids was maintained for at least 20 h of incubation (Grootegoed et al., 1982b). These observations indicate that developing germ cells maintain a specific pattern of synthetic activities in vitro in the absence of Sertoli cells.

In vivo activities of Sertoli cells will be changed after hypophysectomy, because Sertoli cells are target cells for FSH and testosterone. It could be suggested that activities of germ cells are also directly regulated by hormones, because pachytene spermatocytes and round spermatids contain adenylcyclase, cAMP-dependent protein kinase and phosphodiesterase, enzymes involved in cAMP-mediated modulation of cellular activities (Adamo et al., 1980a,b; Gordeladze et al., 1981; Rossi et al., 1982). However, in rats no changes were observed in the pattern of RNA synthesis and RNA processing by pachytene spermatocytes at 64 h after hypophysectomy (Grootegoed et al., 1979). Moreover, it has been shown that after hypophysectomy spermatogenesis was carried out at a normal rate (Clermont & Harvey, 1965). These observations indicate that the absence of FSH and testosterone, and impairment of Sertoli

cell function following hypophysectomy does not induce qualitative changes in spermatocytes. In contrast, after hypophysectomy of rats the number of RNA-synthesizing spermatocytes decreased (Grootegoed et al., 1979) and the number of degenerating cells increased (Russell & Clermont, 1977).
In vitro a positive quantitative effect of pyruvate and lactate on RNA - and protein synthesis of isolated pachytene spermatocytes was observed (Jutte et al., 1981) and it was postulated that synthetic activities of pachytene spermatocytes and round spermatids are supported by supply of pyruvate and lactate from Sertoli cells (Jutte et al., 1982). The metabolism of glucose and the secretion of pyruvate and lactate by Sertoli cells was stimulated by FSH in vitro (Jutte et al., 1982). The amount of proper energy substrates available to germ cells in the spermatogenic micro-environment in vivo may be reduced after hypophysectomy, leading to an inhibition of energy-requiring processes in spermatocytes and spermatids. This may explain why synthetic activities of developing germ cells show rapid quantitative changes, but no qualitative changes after hypophysectomy. In addition to pyruvate and lactate, other factors from Sertoli cells may be involved in the action of Sertoli cells upon germ cells, but a proper supply of pyruvate and lactate to pachytene spermatocytes and round spermatids is probably a prerequisite for other interactions between Sertoli cells and germ cells to occur.
In summary, isolated pachytene spermatocytes maintain a specific pattern of protein synthesis during incubation for 24 h in a chemically defined medium, fortified with pyruvate and lactate. Therefore, within this period, the presence of Sertoli cells may be required to support an optimal rate of synthetic activities in spermatocytes, rather than to modulate these activities.

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Appendix Paper II

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Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids

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Summary. Spermatocytes and round spermatids were isolated from rat testis and the effects of addition of 3·3 mM-glucose and 6 mM-DL-lactate to the incubation medium on the morphology, oxygen consumption and incorporation of uridine and amino acids of these cells were investigated. Addition of lactate to isolated germ cells increased O_2 consumption 1·8-fold and incorporation of precursors of RNA and protein by at least 5-fold. The amino acid incorporation into spermatocytes and spermatids was irreversibly decreased after a preincubation for at least 5 h or 70 min respectively in the absence of lactate, indicating degeneration of the germ cells. In the presence of lactate, however, spermatocytes maintained their morphological integrity for at least 24 h. Addition of glucose to isolated germ cells had no effect on any of the parameters investigated. It is concluded that isolated spermatocytes and round spermatids may utilize lactate, perhaps secreted by Sertoli cells, as the main source of energy.

Introduction

Germ cells in mammalian testes develop in a tubular environment which is controlled to a high degree by Sertoli cells. It is still unknown, however, which Sertoli cell products are important for germ cells. Glucose is known to be very important for the survival of germ cells in the testis. Inhibition of glucose transport by injections of 5-thio-D-glucose (Zysk, Bushway, Whistler & Carlton, 1975; Lobl & Porteus, 1978; Basu, Ramakrishnan, Prasannan, Rama Sarma & Sundaresan, 1979; Majumdar *et al.*, 1979; Majumdar & Udelsman, 1979) and induction of acute hypoglycaemia (Mancini, Penhos, Izquierdo & Heinrich, 1960) result in degeneration of germ cells. In isolated testicular tissue from adult rats, the addition of glucose to the incubation medium resulted in increased RNA synthesis (Hollinger & Hwang, 1972), protein synthesis (Means & Hall, 1968b; Davis, 1969; Hollinger & Hwang, 1972), ATP levels (Means & Hall, 1968a) and oxygen consumption (Tepperman, Tepperman & Dick, 1949; Serfaty & Boyer, 1956; Gomes, 1971). Testicular protein synthesis was increased 7-fold by glucose while in 16 other tissues from adult rats the stimulation was less than 1·5 times (Davis, 1969). Radioautographic analysis of testicular tissue incubated with [^{3}H]lysine has shown that addition of glucose increases especially incorporation of this precursor into protein of pachytene spermatocytes and spermatids (Davis, 1969). No effects of glucose on metabolic activities in testicular tissue have been observed, however, when the number of germ cells was low, as in immature rats (Means & Hall, 1968a, b), following hypophysectomy (Means & Hall, 1968b) or after induction of experimental cryptorchidism (Davis, 1969; Gomes, 1971). Hence, it was concluded that pachytene spermatocytes and spermatids are most dependent on a proper supply of glucose. On the other hand, the effect of glucose on isolated spermatocytes

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and spermatids is small, i.e. approximately 1·5-fold stimulation of amino acid incorporation (Nakamura & Hall, 1976, 1977; Nakamura, Romrell & Hall, 1978). These results suggest a discrepancy between the effects of glucose on germ cells *in vivo* and *in vitro*. In our experiments we observed a pronounced positive effect of lactate and pyruvate on isolated germ cells in media containing glucose. Therefore, we have studied the effects of glucose and lactate on different metabolic activities in isolated germ cells.

Materials and Methods

Cell isolation

Germ cell suspensions were prepared enzymically from testicular tissue of immature rats (Wistar strain, substrain R-Amsterdam, age 30–35 days, body weight 70–80 g) (Romrell, Bellvé & Fawcett, 1976; Bellvé *et al.*, 1977a; Bellvé, Millette, Bhatnagar & O'Brien, 1977b). Pachytene spermatocytes and round spermatids were isolated by velocity sedimentation of the cell suspensions in non-linear albumin gradients (1·0–3·2%) at unit gravity during 70 min at room temperature (Grootegoed, Grollé-Hey, Rommerts & van der Molen, 1977). Cells were counted in a haemocytometer. Isolation medium and incubation medium were essentially Hanks' Balanced Salt Solution (Hanks & Wallace, 1949) and Eagle's Minimal Essential Medium (Flow Laboratories Ltd, Irvine, Ayrshire, Scotland, U.K.). Both media were modified with an increased amount of KCl (56·9 mM) and the osmolarity was adjusted by lowering the NaCl concentration (Grootegoed *et al.*, 1977). The isolation medium was supplemented with 6 mM-sodium-DL-lactate (Sigma; 50% L-lactate). When indicated in the text, lactate and/or glucose were added to the incubation medium to a final concentration of 6 mM-DL-lactate and 3·3 mM-glucose.

Sertoli cells were obtained from 26-day-old rats which had been irradiated *in utero* on Day 19 of gestation with 150 rad (Beaumont, 1960). As a result of this treatment the testes were depleted of germ cells. Testes of these irradiated rats were treated with collagenase (0·5 mg/ml) during 20 min at 32°C to obtain tubules free from myoid and interstitial cells. These tubules were fragmented with a Dounce homogenizer (Fritz, Rommerts, Louis & Dorrington, 1976). All cells were incubated at 32°C.

RNA and protein synthesis

Isolated germ cells were incubated in the incubation medium described above, containing labelled precursors for RNA and protein, during 2 h at 32°C under a humidified atmosphere of 5% CO_2 in air. On several occasions incubation with radioactively labelled precursors was preceded by a preincubation in the absence of this radioactivity. The incubations were stopped by cooling to 4°C and addition of cold 0·9% (w/v) NaCl with either 0·5 mM-uridine and 7 mM-leucine or 6·7 mM-methionine. Cells were filtered over 0·2 μm filters (Sartorius) and washed with 0·9% (w/v) NaCl. Subsequently the cells were lysed and macromolecules were precipitated with 10% (w/v) trichloroacetic acid for 10 min. The precipitate was washed with 10% (w/v) trichloroacetic acid, to remove non-incorporated precursor, and then with 70% (v/v) ethanol. The precipitate was dissolved in 500 μl 1 M-NaOH and radioactivity in the samples was counted after addition of 8 ml Picofluor (Packard).

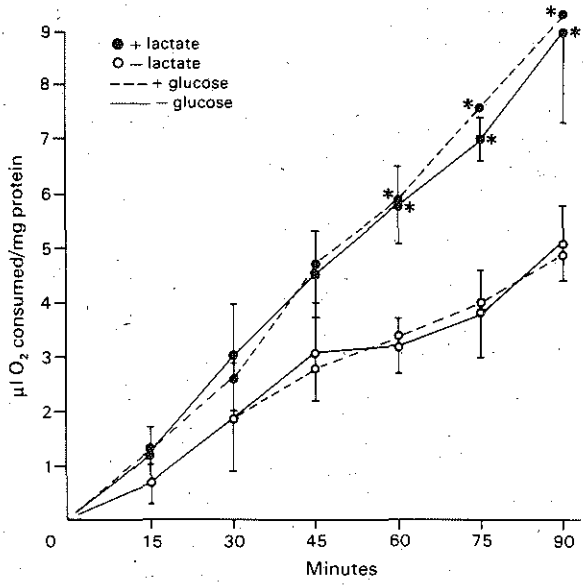
Oxygen consumption

Isolated germ cells were incubated in phosphate-buffered saline (Dulbecco & Vogt, 1954), supplemented with vitamins and amino acids as present in the incubation medium and, when indicated in the text, 6 mM-DL-lactate and/or 3·3 mM-glucose were added. Oxygen consumption was measured in a Warburg apparatus (Umbreit, Burris & Stauffer, 1964).

Results

The morphology (examined with phase-contrast microscopy) of isolated spermatocytes incubated for 24 h in a medium containing 3·3 mM-glucose and 6 mM-DL-lactate or 1 mM-pyruvate, was very similar to that of freshly isolated germ cells. In contrast, the cells cultured without added lactate were degenerate, with translucent cytoplasm and clumped chromatin. Isolated spermatids cultured without lactate became degenerate after several hours. Isolated spermatocytes and spermatids therefore appear to require lactate more than glucose.

The effect of lactate was also demonstrated in short-term experiments. Lactate stimulated oxygen consumption of freshly isolated spermatocytes and spermatids both in the presence and absence of glucose (Text-fig. 1). Lactate had no significant effect, however, on the oxygen consumption of Sertoli cells. Uridine and leucine incorporation into isolated spermatocytes and spermatids was increased at least 5-fold by the addition of lactate, whereas glucose had no significant effect (Table 1). This effect was demonstrated after 60 min preincubation with lactate. In Sertoli cells, addition of lactate with or without glucose caused a 2-fold maximal stimulation of leucine incorporation.



Text-fig. 1. The oxygen consumption by isolated germ cells (37% spermatocytes, 53% round spermatids, 4% other germ cells and 6% somatic cells) from rat testes incubated with (——) or without (——) glucose and with (●) or without (○) lactate. The values are the means obtained with 4 different cell preparations, and vertical bars indicate the s.d. * Significantly different from values without lactate, $P < 0{\cdot}01$ (Student's t test).

Incubation of germ cells in the absence of lactate for short periods did not cause an irreversible change of the capacity to incorporate amino acids. However, after a preincubation period without lactate of at least 5 h and 70 min for spermatocytes and spermatids respectively, the incorporation of methionine was not restored after addition of lactate (Table 2).

Table 1. Incorporation of [^{3}H]uridine and [^{14}C]leucine into isolated germ cells from rat testes

Substrate in incubation medium		Incorporation	
3·3 mM-glucose	6 mM-DL-lactate	[^{3}H]Uridine (nCi/10^6 cells)	[^{14}C]Leucine ($\times 10^{-1}$ nCi/10^6 cells)
–	–	5·5 ± 3·6	1·3 ± 1·0
+	–	21·8 ± 15·5	2·0 ± 1·6
–	+	91·8 ± 24·5*	17·4 ± 7·2*
+	+	125·5 ± 50·9*	20·3 ± 6·7*

The 5×10^5 cells (55–88% spermatocytes, 1–23% round spermatids, 9–15% other germ cells and 2–9% somatic cells) were incubated for 2 h in 1 ml incubation medium containing 5 μCi [5-^{3}H]uridine (final sp. act. 5 mCi/mmol) and 0·6 μCi L-[U-^{14}C]leucine (final sp. act. 12 mCi/mmol) after preincubation for 60 min. Radioactivity in trichloroacetic acid-precipitable material was estimated. Values are mean ± s.d. obtained with 4 different cell preparations.

* Significantly different from incorporation in the absence of glucose and lactate, $P < 0{\cdot}01$ (paired Student's t test).

Table 2. Effect of preincubation without lactate on incorporation of methionine into isolated germ cells from rat testes

Lactate in medium		[^{35}S]Methionine incorporation (nCi/10^6 cells)	
Preincubation*	Incubation	Spermatocytes	Spermatids
–	–	5·7 ± 4·0	0·8 ± 0·3
–	+	18·8 ± 6·6	2·8 ± 1·0
+	+	31·6 ± 10·7	4·9 ± 2·1

The 5×10^5 cells of a spermatocyte fraction (80% spermatocytes, 6–8% round spermatids, 8–12% other germ cells and 4–5% somatic cells) or 10^6 cells from a spermatid fraction (15–20% spermatocytes, 70–76% round spermatids, 6–9% other germ cells and 1–4% somatic cells) were incubated for 2 h in 200 μl incubation medium containing 10 μCi L-[^{35}S]methionine (final sp. act. 20 Ci/mmol) in tubes shaken at 120 oscillations/min. The incubation medium contained 3·3 mM-glucose and, as indicated, 6 mM-DL-lactate. Values are mean ± s.d. for 3 different cell preparations.

* 5–7 h for spermatocytes and 70–90 min for spermatids.

Discussion

The present results show that in germ cells isolated from rat testes exogenous lactate is needed for metabolic activities, as shown by oxygen consumption and RNA and protein synthesis. The effects of the absence of lactate were immediate and were measurable in short-term incubations. When the germ cells were incubated in the absence of lactate for several hours, the decreased metabolic activity was not completely restored after addition of lactate and therefore the cells appear to have degenerated. Degeneration of isolated spermatocytes and round spermatids was readily apparent with phase-contrast microscopy after an incubation without lactate for 24 h. A small percentage of Sertoli cells was always present in our germ cell preparations, but the effects of addition of lactate to germ cell cultures could not be ascribed to contaminating Sertoli cells.

The present observations are very relevant for investigations with male germ cells *in vitro*. The isolation and incubation of these cells have been performed by many investigators using media that did not contain lactate or pyruvate. For example, protein synthesis in isolated germ cells (Nakamura *et al.* 1978) and permeability of the plasma membrane of isolated germ cells (Lee, 1974) have been measured at 32°C and 37°C in media which contained no other substrate than glucose. It is very likely that the observations made in these experiments were markedly influenced by degeneration of the germ cells due to the absence of a proper substrate.

The fact that glucose cannot support metabolic activities of germ cells may be due either to a block in glucose transport or to a block in glycolysis and the hexose monophosphate shunt. It has been demonstrated that the X chromosome is inactive throughout meiotic prophase (Monesi, 1965) and therefore the specific activity of the X-linked iso-enzyme of the glycolytic enzyme phosphoglycerate kinase (PGK) in germ cells may be rather low. It is not possible to conclude, however, that a block in glycolysis in germ cells is caused by the fact that PGK is X-linked because an autosome-linked PGK iso-enzyme, present in testicular tissue, is probably active in spermatocytes and spermatids (VandeBerg, Cooper & Close, 1973, 1976). Similarly, the enzyme glucose-6-phosphate dehydrogenase is X-linked, but a block of the hexose monophosphate shunt in germ cells may be prevented by the activity in these cells of an autosomally coded iso-enzyme demonstrated in testicular tissue (Brock, 1977). There is no definite proof, however, that the enzymes mentioned above or other enzymes involved in glucose metabolism are fully active in spermatocytes and spermatids. It is, therefore, still possible that glucose metabolism is blocked in these cells by inactivity of these enzymes. However, protein synthesis in isolated spermatocytes is not inhibited by the presence of 5-thio-D-glucose (Nakamura & Hall, 1977), an inhibitor of transport of D-glucose (Whistler & Lake, 1972). This observation supports the idea that spermatocytes do not depend on an active glucose transport mechanism.

In pachytene spermatocytes and in spermatids a specific iso-enzyme of lactate dehydrogenase (LDH-X) is present (Meistrich, Trostle, Frapart & Erickson, 1977). It has been shown that testicular LDH-X catalyses preferentially lactate oxidation and is localized in cytosol and mitochondria (Blanco, Burgos, Gerez de Burgos & Montamat, 1976; Montamat & Blanco, 1976). This, as well as the localization of mitochondria in early round spermatids close to the cell surface (Clermont & Rambourg, 1978), may indicate that germ cells are specialized to use exogenous lactate efficiently. Spermatocytes in contact with Sertoli cells in cultures of seminiferous tubule fragments can survive for more than 5 days in a medium with glucose and without lactate (Palombi *et al.*, 1979). The survival of germ cells in this system may be explained by the secretion of lactate by Sertoli cells, because we have observed lactate production by Sertoli cells in culture ($2{\cdot}2 \pm 0{\cdot}8$ μmol/mg protein per day). The effects of glucose on testicular tissue (see 'Introduction') may therefore largely reflect a stimulation of germ cells by lactate produced by Sertoli cells.

The dependence of male germ cells on Sertoli cells is not unique, because a similar relation exists between female germ cells and follicular cells. Granulosa cells have been shown to produce pyruvate (Donahue & Stern, 1968). Isolated mouse and rat oocytes use pyruvate or lactate as an energy source (Biggers, Whittingham & Donahue, 1967; Zeilmaker & Verhamme, 1974; Hillensjö, Hamberger & Ahrén, 1975; Eppig, 1976), but growing oocytes can survive in the absence of pyruvate when cultured in the presence of follicular granulosa cells (Baran & Bachvarova, 1977; Eppig, 1977; Bachvarova, Baran & Tejblum, 1980). It remains to be demonstrated whether lactate and/or pyruvate represent an important intermediate for the interaction between Sertoli cells or granulosa cells and germ cells *in vivo*.

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Appendix Paper III

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Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells

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Summary. During incubation of fragments of seminiferous tubules in the absence of glucose, pachytene spermatocytes and round spermatids died within 24 h, while Sertoli cells were still viable. The germ cells survived for at least 72 h in seminiferous tubule fragments which were incubated in the presence of glucose. Lactate rather than glucose is essential for [^{3}H]uridine incorporation and survival of isolated pachytene spermatocytes. However, if the spermatocytes were incubated in the presence of Sertoli cells, glucose maintained the incorporation of [^{3}H]uridine into the germ cells. Sertoli cells secreted lactate in the presence of glucose and the lactate secretion was stimulated 2–4-fold by FSH. It is concluded that the activity and survival of pachytene spermatocytes *in vitro* can be regulated by the supply of lactate from Sertoli cells.

Introduction

In mammalian testes the walls of the seminiferous tubules are lined by Sertoli cells which are connected by tight junctions and thus form a barrier between the intratubular (adluminal) compartment and the extratubular environment (blood–testis barrier). The composition of the tubular fluid differs from the composition of lymph fluid and blood plasma (Setchell & Waites, 1975). Early primary spermatocytes move across the blood–testis barrier and further germ cell development occurs in the adluminal compartment of the seminiferous tubules.

In the adluminal compartment Sertoli cells may control the composition of the fluid which surrounds the germ cells during development. Sertoli cells are target cells for FSH and testosterone (Fritz, 1978) and these hormones may therefore have an effect on the tubular micro-environment and thus on developing germ cells. Several compounds are known to be secreted by Sertoli cells, e.g. androgen binding protein (Fritz, Rommerts, Louis & Dorrington, 1976), transferrin (Skinner & Griswold, 1980), plasminogen activator (Lacroix & Fritz, 1980), glycoproteins (Galdieri, Zani & Stefanini, 1981), sulphoproteins (Elkington & Fritz, 1980) and *myo*-inositol (Robinson & Fritz, 1979). However, none of these compounds has been shown to affect germ cells. We have reported previously that RNA and protein synthesis and oxygen consumption of isolated spermatocytes and spermatids were stimulated by exogenous lactate (Jutte *et al.*, 1981a; Jutte, Grootegoed, Rommerts & van der Molen, 1981b). These results have been confirmed by the observation that protein synthesis in round spermatids was stimulated by lactate (Nakamura, Hino, Yasumasu & Kato, 1981). The capacity of isolated germ cells to use glucose as an energy source appears to be very low. Glucose, however, is essential for maintenance of spermatogenesis *in vivo* (Mancini, Penhos, Izquierdo & Heinrich, 1960; Zysk,

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Bushway, Whistler & Carlton, 1975). It is possible that the Sertoli cells convert glucose to a substrate which is used by germ cells, and we have investigated whether Sertoli cells can influence the activity and survival of germ cells via the secretion of lactate.

Materials and Methods

Isolation of seminiferous tubules

Testes from immature rats (Wistar, substrain R-Amsterdam, age 30–35 days, body weight 70–80 g) were decapsulated and treated with collagenase (CLS-I; Worthington, Freehold, New Jersey, U.S.A.; 10 mg in 20 ml) in isolation medium (Jutte *et al.*, 1981b) in a 100 ml Erlenmeyer flask during 20 min at 32°C in a shaking waterbath (120 cycles per min). Tubules released by the enzyme treatment were washed three times by sedimentation at unit gravity in isolation medium. When used for culture, the tubules were also washed three times in incubation medium.

Isolation of pachytene spermatocytes

Isolated seminiferous tubules were treated for 15 min with isolation medium containing trypsin (TRL; Worthington; 10 mg in 20 ml) and deoxyribonuclease (DN-Cl; Sigma, St Louis, Missouri, U.S.A.; 20 μg in 20 ml), using the conditions described above, followed by addition of trypsin inhibitor (Sigma; 10 mg in 20 ml) and bovine serum albumin (Sigma, fraction V; final concentration 0·5% w/v). Subsequently, the tubules were dispersed during 3 min with a 1 ml measuring pipette (i.d. 2·5 mm) and the cell suspension was filtered through a nylon filter (60 μm pore size) to remove tubular fragments. A fraction of middle–late pachytene spermatocytes (72% middle–late pachytene spermatocytes, 10% early primary spermatocytes, 14–17% other germ cells and unidentified cells, 1–4% somatic cells) was obtained by sedimentation of the cell suspension at unit gravity for 70 min at 22°C in a non-linear albumin gradient (1–3·2% w/v) (Grootegoed, Grollé-Hey, Rommerts & van der Molen, 1977). The cells were washed three times in incubation medium without glucose before they were used for incubation.

Isolation of Sertoli cells

Sertoli cells were obtained as follows. Isolated seminiferous tubules were fragmented with a Dounce homogenizer (Fritz *et al.*, 1976) and washed three times in incubation medium without glucose. The fragments were incubated without glucose for 1 day, followed by an incubation period of 4–5 days in the presence of 3·3 mM-glucose and 1% (v/v) fetal calf serum (Gibco, Glasgow, Scotland). Pachytene spermatocytes and more mature germ cells were killed during incubation in the absence of glucose and could easily be aspirated from these cultures.

In one series of experiments, as indicated in the text, Sertoli cells were isolated from testes of rats irradiated *in utero* with 150 rad on Day 19 of gestation (Beaumont, 1960). The germ cell-depleted testes of these rats at 24–29 days of age were treated with collagenase, as described above, to obtain Sertoli cell aggregates which were fragmented with a Dounce homogenizer.

Incubation conditions

Cells or tubules were incubated in incubation medium (Jutte *et al.*, 1981b) at 32°C under a humidified atmosphere of 5% CO_2 in air. Glucose and lactate concentrations are given in the 'Results'.

Co-culture of Sertoli cells and pachytene spermatocytes

Sertoli cell monolayers, cultured in 24-well tissue culture plates for 4–5 days, were washed once with medium without glucose and twice with medium containing the required glucose

concentration. Subsequently, $0{\cdot}25 \times 10^6$ pachytene spermatocytes were added to each well (2 cm^2). After 30 min, 2 μCi [5-^{3}H]uridine/ml were added (final sp. act. 5 Ci/mmol). After another 2 h the labelling was stopped with cold 0·9% (w/v) NaCl containing 0·57 mM-uridine. The germ cells were removed from the Sertoli cell monolayer by gentle pipetting with a Pasteur pipette and the amount of [^{3}H]uridine incorporated into RNA of the germ cells was determined by trichloroacetic acid precipitation of RNA on filters (Jutte *et al.*, 1981b). Filters and precipitate were dissolved in 8 ml Filtercount (Packard). Duplicate or triplicate incubations were performed for all conditions. Parallel incubations of Sertoli cells without germ cells were performed to estimate the concentration of lactate in the medium and the amount of cellular protein.

Estimation of lactate and glucose

Seminiferous tubules or Sertoli cells were preincubated for 1 day in 2 ml incubation medium, unless stated otherwise. This medium contained 5 μg FSH/ml (NIH-FSH-S12, 1·25 U/mg, was a gift from the Endocrinology Study Section, NIH, Bethesda, U.S.A.) and/or 200 ng testosterone/ml or the solvents. Concentrated (×100) stock solutions of FSH and testosterone were prepared in 0·9% (w/v) NaCl containing 0·1% albumin (Povite, Amsterdam, Holland) and in 10% (v/v) ethanol respectively.

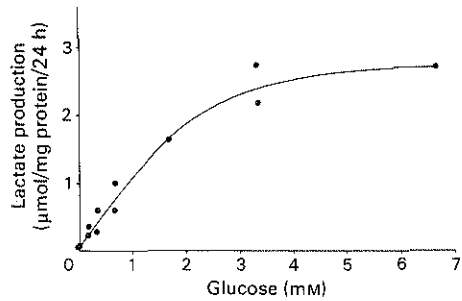
The medium was removed and the cells were washed once with incubation medium. Subsequently, the cells were incubated for 24 h in 2 ml fresh incubation medium containing FSH and/or testosterone and glucose (0–6·6 mM). The medium from the 2nd day of culture was collected, centrifuged to remove unattached cells and kept frozen at −20°C for not more than 1 week until lactate and/or glucose were determined. The attached and unattached cells were collected and lysed in 1 M-NaOH and protein was estimated (Lowry, Rosebrough, Farr & Randall, 1951). Lactate was estimated enzymically, using lactate dehydrogenase (Hohorst, 1970). Glucose was estimated using enzymic conversion with hexokinase and glucose-6-phosphate dehydrogenase (Schmidt, 1961). In all experiments single estimations of lactate and glucose were performed for duplicate incubations of Sertoli cells or seminiferous tubules.

DNA flow cytometry

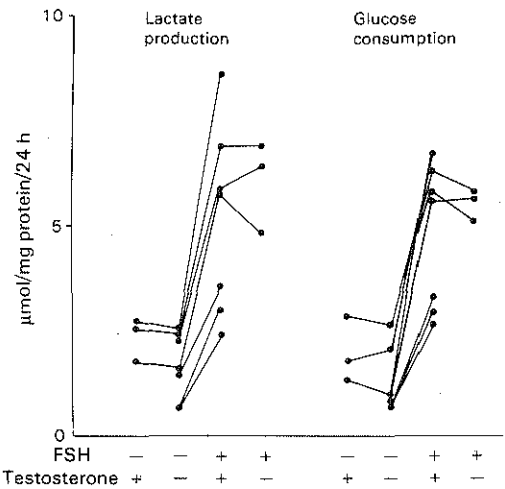
Seminiferous tubules cultured for 1 day without or with glucose (3·3 mM), followed by 2 days of culture in the presence of glucose, were treated with 0·25% trypsin (Difco, Detroit, Michigan, U.S.A.; 1:250) in phosphate-buffered saline (Dulbecco & Vogt, 1954) for 20 min at 37°C. After dispersion by pipetting with a Pasteur pipette, a few drops of fetal calf serum (Gibco, Glasgow, Scotland) were added and the cells were washed in 0·9% (w/v) NaCl. The cells were fixed under continuous mixing by slow addition of cold ethanol (96%, v/v) to a final concentration of 70% (v/v) ethanol. After 30 min at room temperature the cells were stored for several weeks at 4°C. Then the suspensions were washed in 0·9% (w/v) NaCl and incubated in 0·4% pepsin in 0·02 M-HCl at 37°C for 15 min. The cells were cooled in ice, centrifuged and incubated for 30 min at room temperature in a mixture of ribonuclease A (Sigma type 1-A; 10 mg/l), ethidiumbromide (BDH, Poole, England; 10 mg/l) and Nonidet P-40 (BDH; 300 μl/l) (Vindeløv, 1977; Clausen & Åbyholm, 1980). The suspension was filtered through a nylon filter (70 μm pore size) and processed through an Ortho Cytofluorograph 50 H (Ortho Instruments, Westwood, U.S.A.). The counting of the fluorescent nuclei was interrupted after the fluorescence in the highest peak had reached a maximal level. Therefore, the distribution of fluorescence over the different peaks was relative.

Results

Seminiferous tubules in culture produced lactate and the lactate production was dependent on the concentration of glucose in the medium (Text-fig. 1). No detectable change in glucose

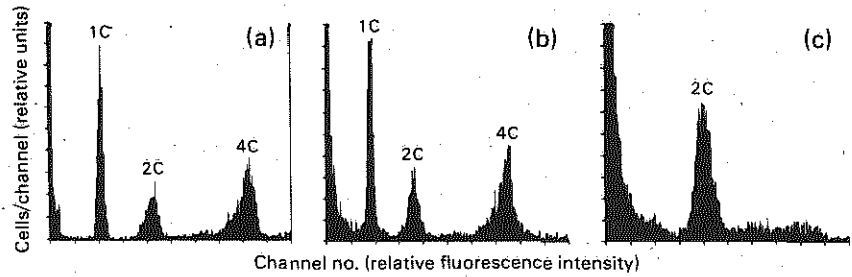


Text-fig. 1. Effect of glucose on the lactate production by rat seminiferous tubules. Fragmented tubules were incubated for 48 h in 7·5 ml medium containing different concentrations of glucose. Lactate was measured in the medium from the 2nd day of incubation (24–48 h). Each point represents the mean of duplicate incubations. Two different cell preparations were used.

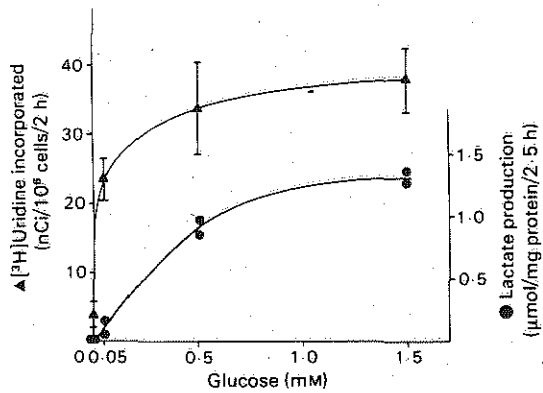


Text-fig. 2. Effect of FSH and testosterone on the lactate production and the glucose consumption of rat Sertoli cells. Sertoli cells obtained from irradiated rats were incubated in medium containing 3·3 mM-glucose. FSH (5 µg/ml) and/or testosterone (200 ng/ml) were present during the incubation from 0 to 48 h. Glucose and lactate concentrations were measured in media collected during 24 h culture (24–48 h). Results obtained with the same cell preparation are connected.

concentration occurred during incubation under these conditions. The lactate secretion by seminiferous tubule fragments from 27-day-old rats was stimulated 3-fold by FSH. Because germ cells may metabolize part of the lactate secreted in seminiferous tubule cultures, we have also studied pure Sertoli cells isolated from irradiated rats. Like seminiferous tubule fragments from intact rats these pure Sertoli cells were able to convert glucose to lactate and the lactate secretion and glucose consumption were stimulated 2–4-fold by a mixture of FSH and testosterone (Text-fig. 2). This stimulation appeared to be caused by FSH under these conditions (Text-fig. 2).



Text-fig. 3. DNA flow cytometric analysis of rat seminiferous tubules cultured for 1 day with or without glucose. Isolated seminiferous tubules were processed for DNA flow cytometric measurements as described in 'Materials and Methods' section. (a) Freshly isolated; (b) incubated for 3 days in the presence of glucose (3·3 mM); (c) incubated for 1 day in the absence of glucose followed by 2 days in the presence of glucose (3·3 mM). Nuclei of primary spermatocytes (4C), spermatids (1C), somatic cells, secondary spermatocytes and spermatogonia (2C) showed different fluorescence after staining of their DNA with ethidium bromide.



Text-fig. 4. The effect of glucose on the incorporation of [^{3}H]uridine into rat spermatocytes, co-cultured with Sertoli cells. Pachytene spermatocytes ($0{\cdot}25 \times 10^6$) were labelled with [5-^{3}H]uridine during 2 h of co-culture with Sertoli cells. The radioactivity incorporations into the spermatocytes are given as means ± s.d., obtained with 3 different germ cell and Sertoli cell preparations. The amount of lactate estimated in parallel incubations of Sertoli cells is given for 2 different cell preparations.

It was shown by phase-contrast microscopy and by cytofluorometric measurements that spermatids and pachytene spermatocytes survived when fragments of seminiferous tubules were incubated for 3 days in the presence of glucose (3·3 mM) (Text-figs 3a, b). However, when seminiferous tubules were incubated without glucose for 1 day, spermatids and spermatocytes were absent (Text-fig. 3c). The Sertoli cells in these tubule cultures survived 1 day of glucose depletion, because after addition of glucose (3·3 mM) on the 2nd day of incubation, the Sertoli cells migrated normally, incorporated leucine (as judged by radioautography) and produced 0·96 ± 0·55 µmol lactate/24 h (compared to controls incubated with glucose, which produced 0·85 ± 0·37 µmol lactate/24 h). Previously we observed that isolated pachytene spermatocytes, incubated in the presence of glucose, did not incorporate significant amounts of [^{3}H]uridine and [^{14}C]leucine (Jutte *et al.*, 1981b). However, the incorporation of [^{3}H]uridine into RNA of isolated spermatocytes incubated in contact with Sertoli cells increased with increasing glucose concentrations (Text-fig. 4). In the absence of glucose, the activity of spermatocytes in

these co-cultures was minimal; maximal activity was reached only in the presence of glucose concentrations greater than 0·5 mM. Lactate production by the Sertoli cells under these conditions was also dependent on the glucose concentration (Text-fig. 4).

Isolated spermatocytes incubated in the presence of 6 mM-DL-lactate and 1·5 mM-glucose incorporated 22·7 ± 4·2 nCi [^{3}H]uridine (10^6 cells/2 h). Spermatocytes incubated on top of Sertoli cells in the presence of 1·5 mM-glucose incorporated 23·1 ± 8·3 nCi [^{3}H]uridine (10^6 cells/2 h) (means ± s.d. of 3 different cell preparations). Therefore, the [^{3}H]uridine incorporation in spermatocytes appeared to be maintained both in the presence of Sertoli cells plus glucose and in the presence of lactate.

Discussion

Isolated pachytene spermatocytes do not survive in the presence of glucose (Jutte *et al.*, 1981b). The present results, however, indicate that RNA synthesis and the integrity of pachytene spermatocytes in cultures of seminiferous tubules and in co-cultures of isolated germ cells and Sertoli cells were maintained by exogenous glucose (Text-figs 3 and 4). When glucose was omitted from these incubations, the pachytene spermatocytes did not survive (Text-fig. 3c). Lactate is known to be essential for survival of germ cells (Jutte *et al.*, 1981b) and the present results indicate that this substrate maintained isolated pachytene spermatocytes as well as did Sertoli cells. Lactate was secreted by the Sertoli cells and this secretion was stimulated by hormones and glucose (Text-figs 1 and 2). These observations support the idea that the activity and survival of pachytene spermatocytes is regulated by Sertoli cells via conversion of glucose to lactate.

Most studies on metabolic pathways in the testis have been performed with mixed cell populations and little is known about metabolic activities in different cell types. It has been suggested that somatic cells in the testis are mainly dependent on lipids for the provision of energy (Free, 1970). We observed that Sertoli cells survived an incubation of 24 h without glucose; Sertoli cells may use lipids under these conditions. Study of the metabolism of glucose by cultures of pure Sertoli cells showed that maximally 2·9% of the glucose utilized was converted to carbon dioxide and 95·8% was converted to anionic compounds, mostly to lactate (Robinson & Fritz, 1981). These observations demonstrate the enormous capacity of Sertoli cells to convert glucose into lactate. In contrast to our observations, Robinson & Fritz (1981) did not observe a stimulatory effect of hormones on lactate production by Sertoli cells. This may be caused by differences in the preparation of the Sertoli cells. The concentration of lactate in tubular fluid *in vivo* is not known, but might be comparable to the concentration of lactate in rete testis fluid (0·65 mM in rams and 0·74 mM in bulls), which is half the concentration present in blood plasma (Setchell, Scott, Voglmayr & Waites, 1969). Glucose appears to be available in the testis because its concentration in the testicular lymph of the rat is as high as the concentration in blood plasma (± 3 mM) (Setchell & Waites, 1975). This does not imply, however, that the Sertoli cells are directly exposed to these concentrations, because at least one other cell type (myoid cells) is interposed between the Sertoli cells and the lymph.

The glucose uptake and/or the glycolysis in isolated germ cells appears to be defective. Lactate oxidation via the Krebs' cycle could supply energy for metabolic activities. Germ cells may be able to maintain an aerobic metabolism, because it has been shown that the oxygen tension in seminiferous tubules is as high as the tension in testis interstitial tissue (Free, Schluntz & Jaffe, 1976). An extreme dependence of germ cells on the activity of the Krebs' cycle is illustrated by in-vivo experiments with rats which were fed inhibitors of the Krebs' cycle. While other tissues were not affected, spermatocytes and spermatids died. This degeneration is probably not caused by a direct effect of the inhibitors on Sertoli cells, because these cells remained in the tubules and were able to support a restored spermatogenesis (Paul, Paul,

Kopko, Bender & Everett, 1953; Novi, 1968; Steinberger & Sud, 1970; Sullivan, Smith & Garman, 1979).

In different stages of germ cell development, a different dependence of germ cells on carbohydrate substrates appears to exist. Round spermatids and pachytene spermatocytes are dependent on a supply of lactate (Jutte *et al.*, 1981b), whereas ejaculated spermatozoa can use glucose or fructose as substrates (Voglmayr, 1975). Therefore, following a stage of dependence on lactate, germ cells may become independent of lactate during or after spermatid elongation.

The present results indicate that lactate from Sertoli cells influences the survival of pachytene spermatocytes and that the lactate production is increased by hormones. Lactate could therefore be an important intermediate for the regulation of the survival of pachytene spermatocytes and round spermatids by hormones.

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Appendix Paper IV

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FSH STIMULATION OF THE SECRETION OF PYRUVATE AND LACTATE BY SERTOLI CELLS MAY BE INVOLVED IN HORMONAL REGULATION OF SPERMATOGENESIS

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SUMMARY

Pyruvate and lactate secretion by Sertoli cells, and the effect of p & l on isolated pachytene spermatocytes and round spermatids were studied. Pure Sertoli cells were obtained from 3-6 week-old rats, which were sterile after prenatal irradiation. The lactate secretion by these Sertoli cells, measured from 24-48 hours after isolation during incubation in the absence of hormones, increased with age of the rats from 3-6 weeks. At all ages investigated, the lactate secretion was enhanced in the presence of FSH plus testosterone, but the stimulation was most pronounced at 4 weeks of age. The increase of the lactate secretion was caused by follicle-stimulating hormone (FSH), whereas testosterone did not exert an effect. Sertoli cells from 4-week-old rats secreted both pyruvate and lactate, which accumulated in the incubation medium in a ratio 1:4. The stimulation of the pyruvate and lactate secretion by FSH was dose-dependent (ED_{50} at approximately 10 ng NIH-FSH-S13/ml). The p & l

secretion was stimulated two-fold by insulin, four-fold by FSH and more than six-fold by bt_2cAMP (in the presence of 3-isobutyl-1-methylxanthine). The effects of FSH and insulin were not additive.

The leucine incorporation into isolated pachytene spermatocytes and round spermatids was stimulated by both exogenous pyruvate and lactate in a dose-dependent way. A maximal rate of leucine incorporation was obtained with 0.2 mM pyruvate or 2 mM L-lactate. Spent medium from incubated Sertoli cells (from 4-week-old rats) stimulated the leucine incorporation into isolated pachytene spermatocytes and round spermatids 4-8-fold. This effect could be explained by the amounts of pyruvate and lactate present in the spent medium. It is concluded that pyruvate and lactate are major secretion products from Sertoli cells, which can support synthetic activities in germ cells, and the present results indicate pyruvate and lactate may play a role in the hormonal regulation of spermatogenesis.

INTRODUCTION

The initiation and maintenance of spermatogenesis in mammalian testes is dependent on the presence of follicle-stimulating hormone (FSH) and testosterone. It has been shown that these hormones exert direct effects on Sertoli cells, whereas it can be concluded from several observations that spermatocytes and spermatids are no target cells for FSH and testosterone (Grootegoed, Peters, Mulder, Rommerts & van der Molen, 1977; Fritz, 1978). Hence, hormonal regulation of spermatogenesis

appears to be mediated by Sertoli cells.

Support for a possible nutritive rôle of Sertoli cells in germ cell development are the recent observations that Sertoli cells secrete p & l at a high rate (Jutte, Grootegoed, Rommerts & van der Molen, 1981; Robinson & Fritz, 1981) and that pachytene spermatocytes and round spermatids require exogenous pyruvate and lactate to carry out energy requiring processes (Jutte et al., 1981a; Jutte, Koolen, Jansen, Grootegoed, Rommerts & van der Molen, 1981; Mita & Hall, 1982). Moreover, the rate of aerobic glucose utilization and p & l production by Sertoli cells from immature rats was found to be increased during incubation in the presence of FSH (Jutte, Jansen, Grootegoed, Rommerts, Clausen & van der Molen, 1982; Jutte, Jansen, Grootegoed, Rommerts & van der Molen, 1982). The present experiments were performed to study the p & l secretion by Sertoli cells in more detail with emphasis on the rôle of FSH during prepubertal testis development, and effects of insulin.

Many unknown componenents secreted by Sertoli cells may be involved in germ cell development. Therefore, we studied also whether p & l are the most important factors, present in spent medium from Sertoli cells, which maintain activities of germ cells during short-term incubations.

MATERIALS AND METHODS

Cell isolation

Germ cells were isolated from immature rats (Wistar, substrain R-Amsterdam, age 30-35 days, body weight 70-80 g) by sedimenta-

tion at unit gravity as described previously (Jutte et al., 1982b). Two germ cell fractions were obtained: a pachytene spermatocyte fraction, which contained mid-late pachytene spermatocytes (80 ± 9%), round spermatids (4 ± 3%), somatic cells (1 ± 1%), other germ cells and unidentified cells (7 ± 4%); a round spermatid fraction, which contained round spermatids (81 ± 9%), early spermatocytes (7 ± 4%), mid pachytene spermatocytes (6 ± 4%), somatic cells (1 ± 1%), other germ cells and unidentified cells (4 ± 2%) (mean ± S.D., n = 13).
Sertoli cells were isolated from 3-6-week-old rats as decribed previously (Jutte et al., 1981a). The rats were irradiated *in utero* on day 19 of gestation to obtain germ cell depleted testes, for the isolation of Sertoli cells not contaminated with germ cells (Beaumont, 1960). Following isolation, the germ cells and Sertoli cells were washed thrice in incubation medium containing 3.3 mM glucose (Jutte et al., 1981a).

Incubation conditions

Sertoli cells (approximately 300 µg of protein) were incubated for 24 h in 2 ml incubation medium (no pyruvate and lactate added) at 32°C under a humidified atmosphere of 5% CO_2 in air, in the absence or presence of FSH (5 µg NIH-FSH-S12, 1.25 U/mg or 0.5 µg/ml NIH-FSH-S13, 15 U/mg, gifts from the Endocrinology Study Section, National Institute of Health, Bethesda, MS, U.S.A.), testosterone (200 ng/ml), dibutyryl cyclic AMP (0.5 mM), 3-isobutyl-1-methylxanthine (MIX, 25 µM) and insulin (5 µg/ml, Sigma, 26.8 U/mg). The medium and additions were renewed after 24 h of incubation and the spent medium from the 24-48 h

incubation period was collected, centrifuged at 1500 g for 10 min to remove a few unattached cells, and stored at 4°C. For some experiments twice the amount of Sertoli cells (approximately 600µg of protein) was incubated as described above, to obtain spent medium (Sertoli cell-conditioned medium).

Pachytene spermatocytes (0.5×10^6 cells) or round spermatids (10^6 cells) were incubated either in 0.5 ml incubation medium plus 0.5 ml Sertoli cell-conditioned medium or in 1 ml incubation medium containing 0-2 mM sodium pyruvate (Boehringer) or 0-4 mM sodium DL-lactate (Sigma, approximately 50% L-lactate).

Incorporation of (^{3}H)leucine into cellular protein was estimated by incubation of germ cells for 2 h in the presence of 5 µCi of (4,5-^{3}H)leucine (final specific radioactivity 20.7 mCi/mmol), following a preincubation of 30 min. The incubations were terminated by addition of cold 0.9% (w/v) NaCl (containing 7.6 mM-leucine) and subsequently the amount of radioactivity incorporated into protein was estimated by precipitation of macromolecules with trichloroacetic acid (Jutte et al., 1981a, 1982b).

The amounts of lactate and pyruvate in the media were estimated enzymically according to Hohorst (1970) and Czok & Lamprecht (1970). The data were statistically analyzed with the Student's t-test.

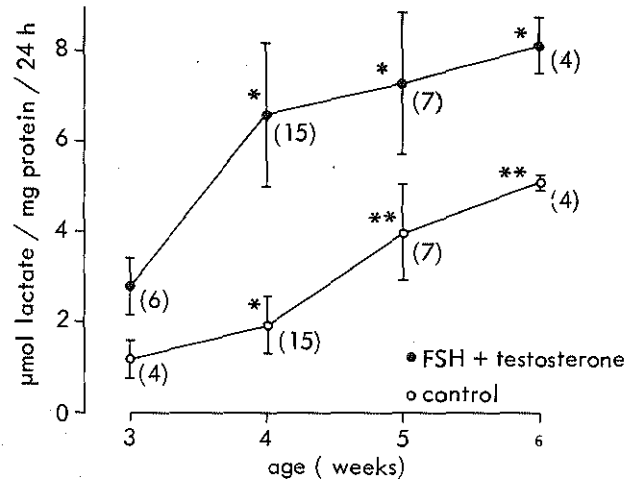

Figure 1. Lactate secretion by Sertoli cells from 3-6-week-old rats.
Sertoli cells obtained from prenatally irradiated rats of different ages were incubated for 24 h in chemically defined medium without further additions (o) or in the presence of FSH (7.5 NIH-FSH-S1 U/ml) and testosterone (200 ng/ml) (•). After 24 h of incubation, the medium was renewed and incubation was continued for another period of 24 h in the presence of the hormones. The amount of lactate present in the spent medium from 24-48 h of incubation was estimated and expressed per mg of cell protein. Results represent means ± S.D. for 4-15 different cell preparations (the number of cell preparations is given between brackets). Each cell preparation was tested in duplicate incubations. At all ages the amount of lactate in the spent medium was significantly higher after incubation in the presence of hormones ($p < 0.01$).
* Significantly different from the lactate secretion by Sertoli cells from 3-week-old rats ($p < 0.05$).
** Significantly different from the lactate secretion by Sertoli cells from 4-week-old rats ($p < 0.05$).

RESULTS

Secretion of p & l by Sertoli cells

From 3 to 6 weeks of age the testes of intact rats become populated with the more advanced germ cell types (pachytene spermatocytes, spermatids) which appear to require p & l . Therefore, we have studied the p & l secretion by Sertoli cells

isolated from 3-6-week -old rats. A preparation of Sertoli cells was obtained from sterile prenatally irradiated rats. The Sertoli cells were incubated in chemically defined medium and lactate was estimated in the spent medium. The following results were obtained:

1) the lactate production in the absence of hormones (basal production, expressed per mg of cell protein) was increased four-fold from 3-6 weeks of age (Fig, 1); 2) the lactate secretion by Sertoli cells from 3-6-week-old rats was increased during incubation in the presence of FSH plus testosterone (Fig. 1), but the ratio stimulated to unstimulated secretion decreased with age as a result of the increased basal production; 3) testosterone did not increase the basal or FSH-stimulated lactate secretion by Sertoli cells from 4-week-old rats (Table 1). The effects of FSH were most pronounced in Sertoli cells from

Table 1. Effects of testosterone and FSH on the lactate secretion by Sertoli cells from 6-week-old rats.

Treatment	lactate secretion (µmol/mg protein/24 h)
No additions	4.98 ± 0.12
testosterone	5.19 ± 0.09
FSH	8.05 ± 0.25*
FSH plus testosterone	8.06 ± 0.71*

The lactate secretion by Sertoli cells from 6-week-old rats was estimated as described in the legend to figure 1. Results represent means ± S.D. for 3 different cell preparations. Each cell preparation was tested in duplicate incubations.
* Significantly different from control and testosterone treated Sertoli cells ($p < 0.01$).

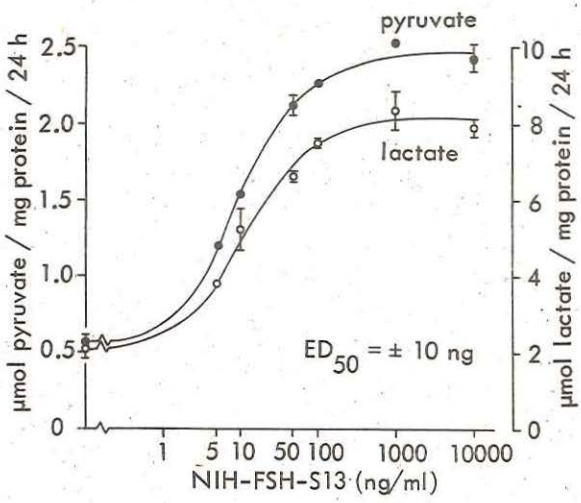

Figure 2. FSH dose-response curve of pyruvate and lactate secretion by Sertoli cells.
Sertoli cells obtained from 4-week-old rats were incubated for 48 h in the presence of different amounts of NIH-FSH-S13. The medium was renewed after 24 h and the amounts of pyruvate and lactate present in the spent medium from 24-48 h of incubation were estimated and expressed per mg cell protein. Results represent means ± range of duplicate incubations.

4-week-old rats and therefore further experiments were performed using these Sertoli cells. The response to FSH of the secretion of both pyruvate and lactate by Sertoli cells from 4-week-old rats was dose-dependent (Fig. 2). Pyruvate and lactate were found in the spent incubation medium in a ratio 1:4. This ratio was the same in the absence or presence of FSH. Half-maximal stimulation of the secretion of both p & l was obtained at approximately 10 ng of NIH-FSH-S13/ml.

Because insulin is involved in glucose metabolism, its effect on the triose secretion by Sertoli cells was investigated. The secretion of pyruvate and lactate by Sertoli cells from 4-week-old rats was stimulated two-fold after 24-48 h of incubation in the presence of insulin, whereas a four-fold stimulation was

found in the presence of FSH plus testosterone (Fig. 3). Insulin did not further increase the rate of p & l secretion obtained in the presence of FSH plus testosterone. Addition of bt_2-cAMP plus the phosphodiesterase inhibitor MIX to isolated Sertoli cells stimulated the p & l secretion six-fold, indicating that the total capacity of Sertoli cells for p & l secretion was larger that was evoked by FSH and insulin (Fig. 3).

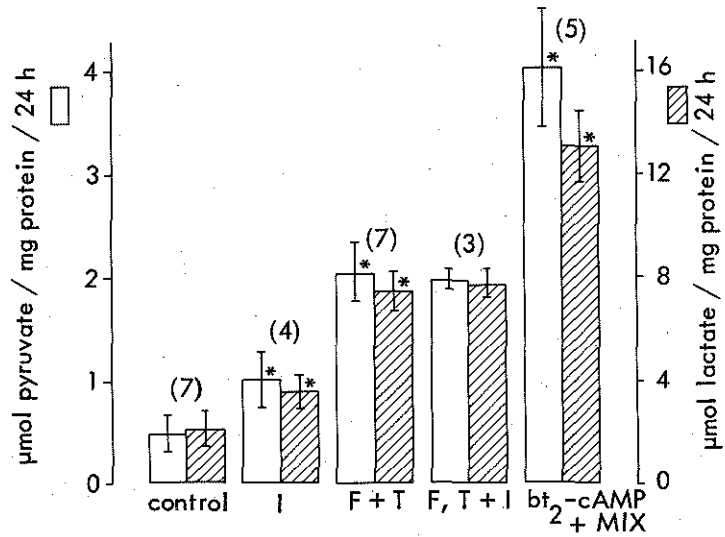

Figure 3. Effects of insulin and bt_2cAMP on the secretion of pyruvate and lactate by Sertoli cells from 4-week-old rats.
Sertoli cells were incubated as described in the legend to Fig. 2 in the presence or absence of insulin (I)(5 µg/ml), FSH (F) (0.5 µg NIH-FSH-S13/ml), testosterone (T) (200 ng/ml) or bt_2cAMP (0.5 mM) and MIX (25 µM). Results represent means ± S.D. for 3-7 different cell preparations (the number of cell preparations is given between brackets). Each cell preparation was tested in duplicate incubations.
* Values significantly different from the adjacent values to the left in the figure ($p < 0.01$).

Effect of p & l on germ cells

The stimulation of (^{3}H)leucine incorporation into isolated pachytene spermatocytes and round spermatids by exogenous lactate and pyruvate was dose-dependent (Fig. 4). Pyruvate was effective at 10-fold lower concentrations than lactate to support a high rate of leucine incorporation, since maximal leucine incorporation was reached at approximately 2 mM L-lactate or 0.2 mM pyruvate for both pachytene spermatocytes and round spermatids during an incubation for 2 hours in the presence of 3.3 mM glucose. Moreover, addition of both L-lactate (3 or 6 mM) and pyruvate (2 mM) to spermatocytes and spermatids did not further increase the leucine incorporation above the level obtained by addition of pyruvate alone (results not shown). This indicates that pyruvate, irrespective of the presence of lactate, can support a high rate of protein synthesis during short-term incubations.

Effect of Sertoli cell-conditioned medium on germ cells

To define the relative importance of p & l to germ cells, as compared to other products secreted by Sertoli cells, we have investigated the effect of spent medium from incubated Sertoli cells on germ cells. Isolated pachytene spermatocytes and round spermatids were incubated for 2 h in Sertoli cell-conditioned medium (1:1 diluted with fresh medium) and (^{3}H)leucine incorporation into the cells was measured. The pmol amounts of incorporated leucine were calculated from the incorporated radioactivity, after correction for an increase of the specific radioactivity of leucine in the spent media, caused by leucine consumption by

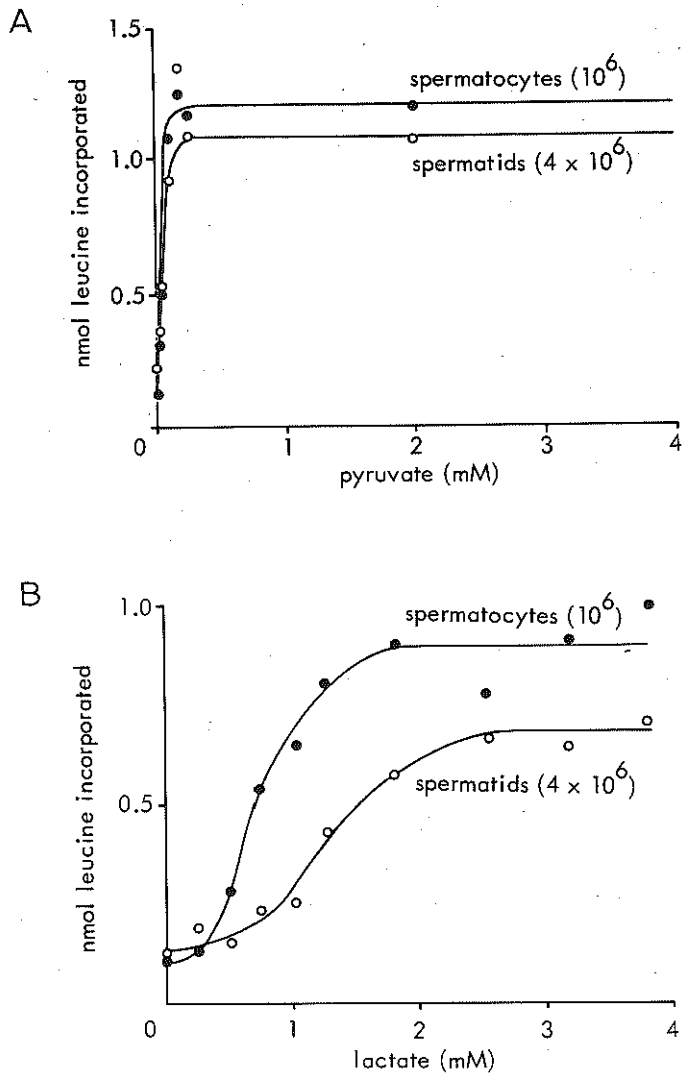

Figure 4. Effect of lactate and pyruvate concentrations on the incorporation of leucine into isolated spermatocytes and spermatids.
Isolated germ cells were incubated for 2 h in incubation medium containing different concentrations of pyruvate (A) or lactate (B) in the presence of 5 µCi (^{3}H)leucine (final spec. act. 20.7 mCi/mmol). Radioactivity incorporated into acid-precipitable material was estimated as described in Material and Methods. The total amount of leucine incorporated was calculated.

Sertoli cells. In the 1:1 diluted conditioned medium this leucine consumption may account for maximally a two-fold increase of the specific radioactivity of leucine and this could result in maximally a two-fold increase in incorporation of (^{3}H)leucine into spermatocytes and spermatids incubated in this medium. The incorporation of leucine into isolated pachytene spermatocytes and round spermatids incubated in conditioned medium was respectively 8- and 4.5-fold (uncorrected 16- and 9-fold) higher than into germ cells incubated in fresh medium (Figure 5). The concentrations of pyruvate and lactate in the Sertoli cell-conditioned media (1:1 diluted with fresh medium) were respectively: 0.16 $\pm$ 0.04 mM and 0.70 $\pm$ 0.19 mM (mean $\pm$ S.D. for 3 different cell preparations) at the start of the incubation with germ cells. From the dose-response curves (Fig. 4) it appears that these p & l concentrations may account for the effect of Sertoli cell-conditioned media on protein synthesis in spermatocytes and spermatids.

DISCUSSION

The results presented indicate that, among many components presumably present in spent medium from Sertoli cells, pyruvate and lactate are major secretion products, which act to maintain a high rate of protein synthesis in isolated spermatocytes and spermatids during short-term incubations. Moreover, pyruvate and lactate production by Sertoli cells were markedly stimulated by FSH, and it is therefore tempting to suggest that p & l may be involved in the effects of FSH on spermatogenesis.

FSH and testosterone are involved in initiation of spermatogenesis

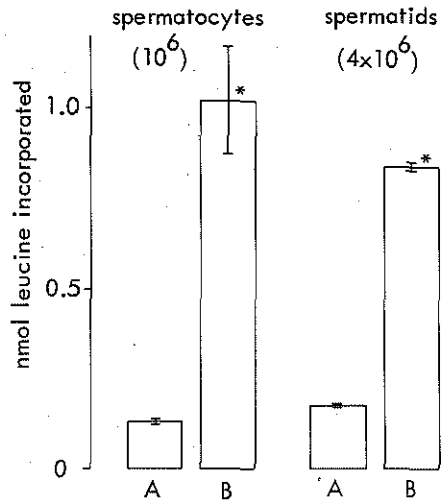

Figure 5. Effect of Sertoli cell-conditioned medium on the incorporation of leucine into spermatocytes and spermatids.
Sertoli cells from 4-week-old rats were incubated in the presence of NIH-FSH-S12 (5 µg/ml) and testosterone (200 ng/ml). Spent media from 24-48 h of incubation were collected (conditioned medium). Isolated germ cells were incubated during 2 h in fresh medium (A), or in a mixture of 0.5 ml fresh medium plus 0.5 ml Sertoli cell-conditioned medium (B). (^{3}H)Leucine incorporation was measured as described in the legend to Fig. 4. The total amount of leucine incorporated into germ cells incubated in conditioned medium was calculated after correction for utilization of leucine by Sertoli cells (see Results). Conditioned media from three different Sertoli cell preparations were tested on six different germ cell preparations (each germ cell preparation was tested in duplicate incubations) and the results are presented as means ± S.D. (n = 3). Incorporation in fresh medium was significantly different from the incorporation in conditioned medium (* $p < 0.01$).

in immature rats or in restoration of a regressed germinal epithelium after long-term hypophysectomy in adult animals, whereas spermatogenesis in adult rats largely can be maintained by testosterone (Steinberger, 1971; Fritz, 1978). Stimulation of Sertoli cell activities by FSH was previously reported to decrease after 20 days of age (Dorrington, Fritz & Armstrong, 1978; Fritz, 1978; Means, Dedman, Tash, Tindall, van Sickle

& Welsh, 1980). In our experiments, the effect of FSH on the p & l secretion by Sertoli cells was most pronounced using Sertoli cells from 4-week-old rats and decreased at higher ages. This may be related to the above mentioned age-dependent pattern of hormonal stimulation of spermatogenesis. The absence of an effect of testosterone on the p & l secretion by Sertoli cells from 4- and 6-week-old rats may indicate that glucose metabolism by Sertoli cells is not involved in the effect of testosterone on spermatogenesis. The results of the present short-term experiments, however, do not exclude that testosterone exerts a long-term effect on glucose metabolism by Sertoli cells in vivo.

If p & l are involved in the effects of FSH on spermatogenesis, the production of p & l in the spermatogenic micro-environment should be a limiting factor for germ cell development, so that production of p & l in the absence of FSH is too low to support development of an optimal number of germ cells. The following variables may influence the amount of p & l present in the germinal epithelium in the absence of FSH.

1) In vivo, transport of p & l from the blood into the seminiferous tubules may overshadow the contribution of Sertoli cells to the amount of p & l in the spermatogenic micro-environment. As far as we know, no data are available on transport of p & l across the blood-testis barrier.
2) At low levels of FSH, the production of p & l in vivo may be stimulated by insulin. It has previously been shown that insulin stimulates transferrin and ABP secretion by Sertoli cells both in the absence and in the presence of FSH (Karl &

Griswold, 1980; Skinner & Griswold, 1982). We observed that insulin did not further enhance p & l secretion, when Sertoli cells were incubated in the presence of FSH. It will be of interest to study the possible long-term effects in vivo of FSH and insulin on carbohydrate metabolism by Sertoli cells.

3) The p & l secretion by isolated Sertoli cells, incubated in the absence of FSH, was increased with age from 3-6 weeks. This may reflect a higher basal rate of glucose metabolism in Sertoli cells from the older animals. On the other hand FSH stimulation of Sertoli cells in vivo may be maintained during incubation, and the basal level of p & l secretion measured in vitro may actually represent a stimulated level.

The production of p & l by Sertoli cells, and the ratio pyruvate to lactate, may be dependent on many conditions which have an effect on cellular NAD^+/NADH ratios through modulation of glucose utilization, citric acid cycle activity etc. To maintain protein synthesis of germ cells, pyruvate was effective at a 10-fold lower concentration than lactate and it seems that secretion of pyruvate by Sertoli cells is rate-limiting for activities of germ cells. However, it is not possible to predict the relative rates of pyruvate and lactate utilization under conditions in vivo. In the spermatogenic micro-environment, lactate may become the limiting substrate for germ cells.

After hypophysectomy many spermatocytes and spermatids die (Clermont & Morgentaler, 1955). It has been reported that after hypophysectomy the rate of development of a small number of surviving germ cells was not changed (Clermont & Harvey, 1965). Moreover, RNA synthesis and processing was unaltered in a part

of the population of advanced germ cells which survived 2-4 days hypophysectomy in immature rats (Grootegoed, van Meerkerk, Rommerts & van der Molen, 1979). Such a quantitative effect on the number of developing germ cells may be related to a lack of substrate required for energy supply. In vivo, stimulation of Sertoli cells by FSH may maintain a high rate of substrate supply, so that development of a maximal number of germ cells can be supported. From the results presented, we conclude that the rate of pyruvate and lactate production by Sertoli cells may be involved in the hormonal regulation of spermatogenesis.

ACKNOWLEDGEMENTS

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