LEYDIG CELLS IN THE TESTIS OF THE RAT

Heterogeneity, development
and similarities with macrophages

Omslag

De foto aan de voorzijde toont een gedeelte van de testis van een volwassen rat, ca. 200x vergroot. In dit stukje testisweefsel is het volgende te onderscheiden. Een deel van een zaadvormend buisje, lopend van linksonder naar rechtsboven, met daarin, vaag zichtbaar, de zaadcellen. Aan de rechterzijde erlangs loopt, als een donkere streep, een bloedvat. Het weefsel eromheen, het interstitium, bevat voornamelijk Leydig cellen (grijze massa) en macrofagen (zichtbaar als donkere vlekken door opname van de kleurstof trypaan blauw).

De compositie aan de achterzijde toont een variëteit aan testispreparaten en histochemische kleuringen.

LEYDIG CELLS IN THE TESTIS OF THE RAT

Heterogeneity, development and similarities with macrophages

LEYDIG CELLEN IN DE TESTIS VAN DE RAT

Heterogeniteit, ontwikkeling en overeenkomsten met macrofagen

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE GENEESKUNDE
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OP GEZAG VAN DE RECTOR MAGNIFICUS
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aan mijn ouders

CONTENTS

APPENDIX PAPERS

I. Molenaar, R., Rommerts, F.F.G. and van der Molen, H.J. (1983). The steroidogenic activity of isolated Leydig cells from mature rats depend on the isolation procedure. Int. J. Androl. 6, 261-274.

II. Molenaar, R., Rommerts, F.F.G. and van der Molen, H.J. (1986). Non-specific esterase: a specific and useful marker enzyme for Leydig cells from mature rats. J. Endocrinol. 108 (3), 329-334.

III. Molenaar, R., de Rooij, D.G., Rommerts, F.F.G., Reuvers, P.J. and van der Molen, H.J. (1985). Specific destruction of Leydig cells in mature rats after in vivo administration of Ethane Dimethyl Sulphonate (EDS). Biol. Reprod. 33, 1213-1222.

IV. Molenaar, R., de Rooij, D.G., Rommerts, F.F.G. and van der Molen, H.J. (1986). Repopulation of Leydig cells in mature rats after selective destruction of the existent Leydig cells with Ethylene Dimethane Sulfonate (EDS) is dependent on LH and not FSH. Endocrinology, in press.

CHAPTER I
GENERAL INTRODUCTION

I.1. SCOPE OF THIS THESIS

Leydig cells are present in the interstitium of the testis and are responsible for the steroid production of the testis. This production is regulated by the pituitary hormone LH and there are indications that it is also under the influence of local factors.

Heterogeneity of cell populations has been reported for a variety of cell types. Factors which can account for this heterogeneity are e.g. differences in the stage of differentiation and differences in localization, whereas in other cases the heterogeneity appeared to be related to "intrinsic" properties of the cells. There are indications that the population of Leydig cells present in the testis is heterogeneous too. Several authors have isolated subpopulations of Leydig cells differing in steroidogenic activities.

Besides Leydig cells the interstitium also contains macrophages, fibroblasts, myoid cells, endothelial cells and mesenchymal cells. The macrophages are found closely associated with Leydig cells and have some properties in common with Leydig cells. This may give rise to a heterogeneity within the population of "Leydig cells".

The differentiation of Leydig cells in the rat shows a biphasic pattern. Two generations of Leydig cells can be discerned: the foetal generation and the adult generation. The adult population of Leydig cells undergoes many changes during sexual development. If this occurs asynchronously, this may result in a heterogeneous population of Leydig cells during this period. Little is known about the turnover of Leydig cells after maturation and it is not clear, therefore, whether and to which extent differentiation of Leydig cells contribute to the heterogeneity of the Leydig cells present in testes from mature

rats.

The precise nature of the precursor cell which gives rise to the adult population of Leydig cells has not been elucidated yet. Mesenchymal cells, fibroblasts, endothelial cells and also macrophages have been mentioned as possible precursor cells.

The work described in this thesis concerns:

1. the heterogeneity of Leydig cells
2. the (dis)similarities between Leydig cells and macrophages in the testis
3. the development of Leydig cells

The heterogeneity of Leydig cells obtained from mature rats and the effect of the isolation procedure on the heterogeneity of the resulting cell preparation was evaluated by comparing different isolation procedures (Chapter II.1.). Attempts to separate Leydig cells into different subpopulations are described in Chapter II.2. Properties of testicular macrophages and Leydig cells were compared using markers specific for Leydig cells or macrophages (Chapter III).

Administration of a chemical compound Ethylene Dimethane Sulfonate (EDS) causes a selective depletion of Leydig cells followed by a regeneration of Leydig cells. This process of regeneration and the role of the gonadotrophic hormones is described in Chapter IV.

The change of the cellular composition of the interstitial tissue as a whole and the changes in properties of Leydig cells in the interstitium during sexual maturation have been described in Chapter V.

In Chapter VI the results are discussed with respect to heterogeneity, origin and development of Leydig cells and a possible role of the Leydig cells in immunosuppression.

I.2. THE TESTIS

In mammals the testes fulfil two functions, viz. the production of spermatozoa, required for sexual reproduction, and the production of the male steroid hormones, the androgens, required for the development and maintenance of spermatogenesis and secondary sex characteristics. Spermatogenesis takes place in the seminiferous tubules, whereas the androgen production occurs in the interstitial tissue, which surrounds the tubules (Fig. I.1.).

The inside of the seminiferous tubules is lined by Sertoli cells which through cytoplasmic extensions surround almost all developing germ cells. The development of germ cells is under the influence of the pituitary hormone, FSH, and the androgen testosterone, both acting via the Sertoli cells.

In the interstitium the Leydig cells are the site of androgen production. The steroid production by the Leydig cells is under the influence of the pituitary hormone LH. There is evidence that Leydig cell function is regulated also by local factors, probably produced by the Sertoli cells (Benahmed et al., 1985) and by still unknown factors specifically present in testicular interstitial fluid (Sharpe & Cooper, 1984; Rommerts et al., 1986).

The testicular interstitium contains, in addition to Leydig cells, other cell types such as macrophages, fibroblasts, myoid cells, endothelial cells and mesenchymal cells. Macrophages are frequently found closely associated with Leydig cells (Connell & Christensen, 1975; Wing & Lin, 1977; Miller et al., 1983) and it has been observed that changes in the morphology of the Leydig cells caused by e.g. cryptorchidism (Bergh, 1985), LH withdrawal (Gondos et al., 1980) or seasonal reproductive inactivity of the testis (Wing & Lin, 1977) occur concomitant with changes in the morphology of the macrophages. Little is known, however, about the function of the testicular macrophages.

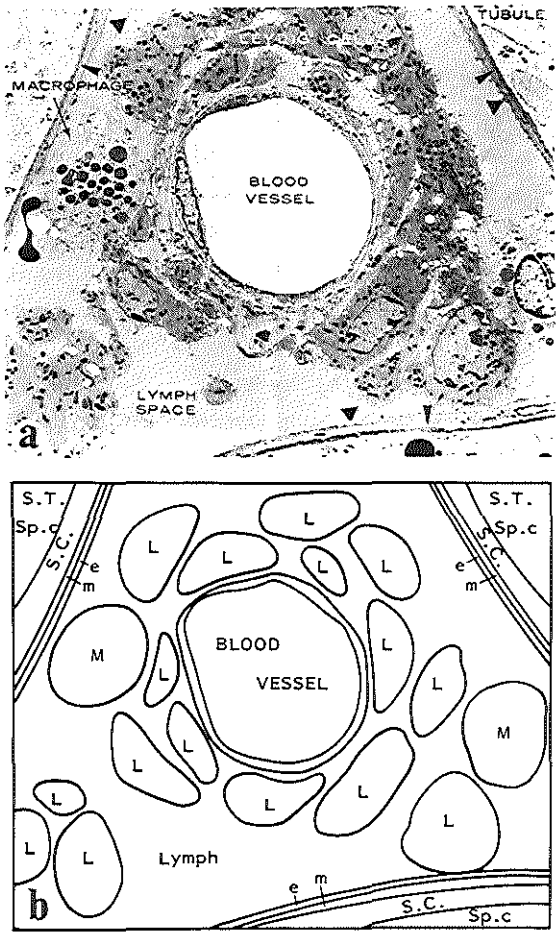


Fig. I.1: Cross-section of a testis from a mature rat.
a. Transmission electron micrograph, x2140 (Clark, 1976).
b. Schematic reflection of a.
The space between the seminiferous tubules (S.T.), which is called the interstitium, contains Leydig cells (L) and macrophages (M) and is filled with lymph. Blood vessels penetrate the interstitium. The vascular wall consists of endothelial cells (in a; e in b). The outside of the tubules is surrounded by a layer of myoid cells (➤ in a; m in b) and a layer of endothelial cells (▶ in a; e in b). The inside of the tubules is lined by Sertoli cells (S.c), which enclose the spermatogenic cells (Sp.c) (b).

This is in contrast to the fibroblasts and myoid cells which appear to be important for the support and the contractibility of the seminiferous tubules, respectively. The endothelial cells in the interstitium form the lining of the blood vessels and lymph space. The mesenchymal cells have been considered as possible precursors for Leydig cells, although macrophages and endothelial cells have been mentioned also as potential precursors of Leydig cells.

I.3. LEYDIG CELLS

I.3.1 Discovery of Leydig cell function

It has been known since antiquity that the testes are responsible for the control of male characteristics. In 1849 Berthold demonstrated by transplantation of testes ectopically into castrated roosters that the effect of testes on male characteristics involved the secretion of a factor from the testes into the blood. The precise source of this factor remained unknown. Only one year later, in 1850, Franz Leydig described for the first time the presence of cells in the interstitium of the testis, which were later named after him "Leydig cells" as follows: "Eine zellenähnliche Masse, welcher Hauptbestandteil sind Körperchen von fettartigem aussehen; sie umlagern helle, bläschenförmige Kerne und ihre halbflüssige Grundmasse mag sich auch wohl zu einer Zellenmembran verdichten, wenigstens zieht bei manchen Säugetieren um den ganzen Körnerhaufen eine scharfe Contur, auch ist bisweilen der ganze Habitus so, dass man von einer fertigen Zelle sprechen kann". Later studies, using various pathological or experimental conditions with regressed tubules but with normal Leydig cells, suggested that the maintenance of male characteristics was correlated with the state of the Leydig cells rather than that of the seminiferous tubules. It was, however, more than one century after the description of the Leydig cells by Franz Leydig that it was actually proven

that the Leydig cells were the main source of the androgens, the hormones which are required for the normal male appearance. This evidence was obtained from the experiments by Baillie et al. (1966) who demonstrated that the activity of the steroidogenic enzyme 3ß-Hydroxy Steroid Dehydrogenase was localized predominantly in the Leydig cells and by Hall et al. (1969) who demonstrated by separation of the seminiferous tubules from the interstitial tissue, that the conversion of cholesterol to androgens occurred only in the interstitial tissue.

I.3.2. Leydig cell morphology

After the general description of the Leydig cell by Franz Leydig in 1850, numerous studies have described the fine structure of the Leydig cell in more detail. Characteristic for the Leydig cell is the presence of a large, round or oval nucleus with a thin rim of peripheral heterochromatin, containing one or two prominent nucleoli, abundant smooth endoplasmic reticulum and mitochondria, often showing tubular cristae, and occasional rough endoplasmic reticulum, Golgi complexes, lysosomes and lipid droplets (Christensen, 1975; Kerr & Sharpe, 1985[b]; Laws et al., 1985).

The structure of Leydig cells in the testes of mammals essentially conforms to this description, but the details can vary (see review Christensen, 1975). For example, the smooth endoplasmic reticulum is more abundant in Leydig cells from guinea pigs, mice and boars than in rat Leydig cells. Mitochondria with tubular cristae are present in the Leydig cells of many species but not very clearly in Leydig cells from humans, guinea pigs or rats. Lipid droplets which are common in Leydig cells from humans, guinea pigs and mice, are uncommon in boars and adult rats. Moreover, Leydig cells can vary largely in size: Leydig cells in the rat are approximately 10µm in diameter, whereas Leydig cells in the human and the boar are approximately 15 and 30 µm in diameter, respectively.

In addition, the appearance of the Leydig cell can vary depending on the season. In most seasonal breeders Leydig cells transform from an "undifferentiated" interstitial cell before the breeding season into an active Leydig cell showing abundant smooth endoplasmic reticulum and mitochondria often with tubular cristae during the breeding period and they regress thereafter loosing their specific Leydig cell characteristics (Wing & Lin, 1977; Hochereau de Reviers & Lincoln, 1978).

I.3.3. Origin and development of Leydig cells

In the mammilian testis the Leydig cells show two or three phases of development. In the human (Mancini et al., 1965); Pelliniemi & Niemi, 1969) and in the pig (van Straaten & Wensing, 1978; van Vorstenbosch et al., 1984) three distinct populations of Leydig cells can be discerned. The first population appears at the time of gonadal sex differentiation in the foetus and regresses thereafter. This population is independent on gonadotrophic hormones. The second population (which is dependent on gonadotrophic hormones) is also transient and is present in the perinatal period. The third wave of Leydig cell differentiation occurs at the onset of puberty, probably under the influence of LH, and results in the final, adult population of Leydig cells. In the rabbit (Gondos et al., 1976), the rat (Lording & De Kretser, 1972) and the mouse (Russo & de Rosas, 1971) only two populations of Leydig cells can be discerned, a perinatal (also called foetal) population and an adult population. In some species (human (Mancini et al., 1963; Chemes et al., 1985) and rabbit (Gondos et al., 1976)) the occurence of the different populations is clearly separated in time. This is in contrast to the situation in the pig (van Straaten & Wensing, 1978) and the rat (Lording & De Kretser, 1972; Tapanainen et al., 1984) which shows an overlap between the perinatal population and the adult population. It is generally believed that the perinatal population arises from a mesenchymal cell (Lording & de

Kretser, 1972; Gondos et al., 1976; van Straaten & Wensing, 1978), but the precise nature of the precursor cell for the adult population, which appears at the onset of puberty, has not been elucidated yet. Various cell types have been mentioned as possible precursor cells: mesenchymal cells (Kerr & Sharpe, 1985[b]), endothelial cells (Laws, 1985), fibroblast-like cells (Mancini et al., 1963; Chemes et al., 1976), macrophages (Clegg & McMillan, 1965) and also dedifferentiated Leydig cells from the foetal population, which may remain in the testis (Prince, 1984).

It is known that the adult population of Leydig cells undergoes several changes during sexual maturation. The cell size as well as the number of Leydig cells increase (Knorr et al., 1970; Tapanainen et al., 1984) and the cells acquire a higher number of LH receptors (Ketelslegers et al., 1978; Clausen et al., 1981). Furthermore the activities of the various steroidogenic enzymes and the metabolic pathways of steroid production show great changes (see review van der Molen & Rommerts, 1981).

Little is known about the turnover of Leydig cells and it is not clear to which extent differentiation and development of Leydig cells in the testis continues after maturation. Experiments from Christensen & Peacock (1980) have demonstrated, however, that prolonged treatment with hCG can lead to a 3-fold increase in the number of Leydig cells in testes from mature rats, which implies that the capacity for cell division and differentiation is still present after maturation.

I.3.4. Steroid production by Leydig cells

The steroid production by the Leydig cells is regulated mainly by the pituitary hormone LH. LH binds to specific membrane receptors, which causes an increase in second messengers (c-AMP and Ca^{2+}), activation of protein kinases, phosphorylation and synthesis of proteins, which ultimately

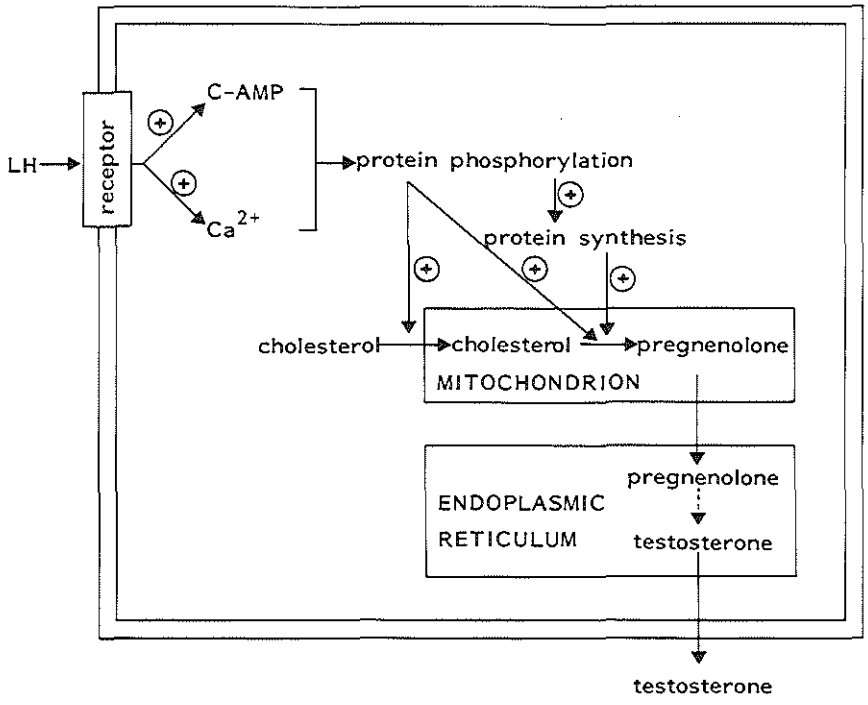


Fig. I.2.: Schematic presentation of the different steps involved in LH-stimulated steroid production in Leydig cells.

results in an increased conversion of cholesterol to pregnenolone (Cooke et al., 1976; Bakker, 1983; Themmen et al., 1986) (Fig. I.2.). The conversion of cholesterol to pregnenolone, the so-called cholesterol side chain cleavage (CSCC) reaction, takes place in the mitochondria catalysed by the cholesterol side chain cleavage enzyme complex (Fig. I.3.) and is generally considered as the rate limiting step in steroid production. Pregnenolone leaks out of the mitochondria and is converted to several other steroids (Fig. I.3.), including testosterone as the main end product in Leydig cells from mature rats.

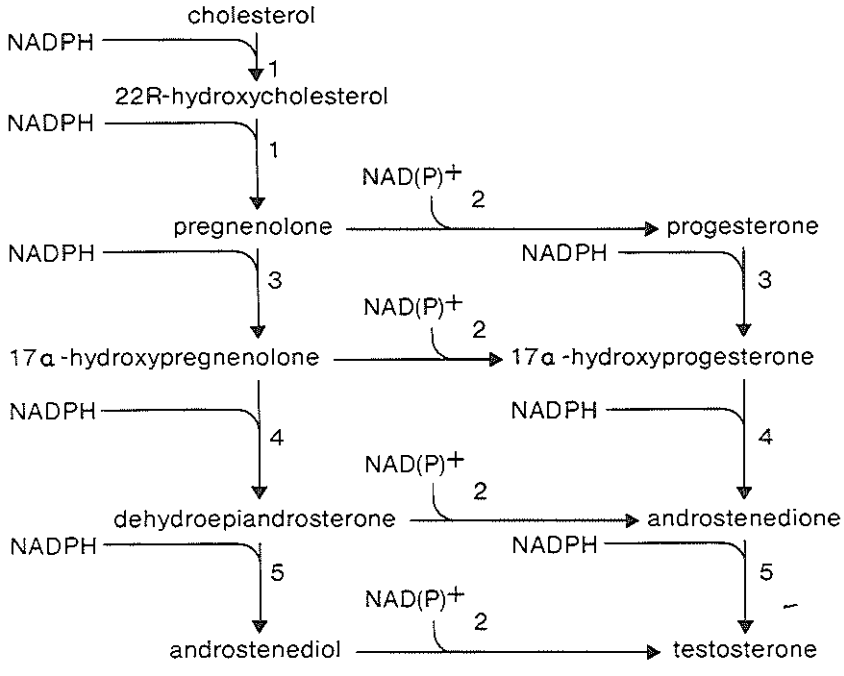


Fig. I.3.: Main steps involved in testosterone biosynthesis.
1 cholesterol side chain cleavage enzyme complex
2 3β-Hydroxy Steroid Dehydrogenase
3 17α-hydroxylase
4 steroid C17-20 lyase
5 17β-Hydroxy Steroid Dehydrogenase

I.4. SIMILARITIES BETWEEN LEYDIG CELLS AND MACROPHAGES

The role of the macrophages in the testis is still not clear, but it has been shown that these cells show activities in other organs which may be related to steroid production. The presence of androstenedione metabolizing enzymes in alveolar and peritoneal macrophages (Milewich et al., 1982,1983.; Lofthus et al., 1984) and of 17α-hydroxylase in adrenal cortex macrophages (Vernon-Roberts, 1969) have been demonstrated. Moreover, a stimulatory effect of macrophages on progesterone

production by granulosa cells in vitro has been observed (Kirsch et al, 1981).

On the other hand isolated Leydig cells show some characteristics which are generally ascribed to macrophages viz. specific binding of lymphocytes (Born & Wekerle, 1981; Rivenson et al., 1981) and production of prostaglandins (Haour et al., 1979; Molcho et al., 1984). Furthermore immunosuppressive activity was found in the testis (Head et al., 1983) and there are indications that the Leydig cells may be responsible for this activity (Born & Wekerle, 1982; Staples et al., 1983). These data suggest that Leydig cells and macrophages may have certain functional properties in common.

I.5. HETEROGENEITY OF CELL POPULATIONS

I.5.1. General aspects of heterogeneity

Cell populations which contain only cells of the same cell type can still be heterogeneous with respect to certain properties.

In some cases the basis for this heterogeneity is known e.g. when the stage of differentiation or the localization of the cells within the tissue are responsible for this heterogeneity. Rapidly dividing tissues and tissues containing cells which show a high turnover, depend on a continuous differentiation of cells which may result in a heterogeneous population. This has been demonstrated for tumours (Buick, 1984; Rubin, 1984;) and in cells belonging to the mononuclear phagocyte system (macrophages) (Dougherty & McBride, 1984; Hofman et al, 1984). Different properties resulting from a difference in localization have been demonstrated in various cell types, such as: hepatocytes, where the transport function of the cells in the periportal region was found to be clearly different from the function of cells in the perivenous region (Groothuis, 1982), macrophages which can express many different

characteristics with respect to antigen expression, metabolic activities, secretory activities (Dougherty & McBride, 1984) depending on the tissue (lung, liver, spleen and peritoneum), and adrenal cells, where the "innerzone" cells are more responsive to ACTH in steroid production than the "outerzone" cells (Cronshaw et al., 1985).

In other cases the heterogeneity seemed to be related to intrinsic properties of the cells. The presence of two populations of cells differing in steroidogenic activities have been demonstrated in the corpus luteum (Ursely & Leymarie, 1979; Hoyer & Niswender, 1985). One population consisted of small cells which produced progesterone at a low rate under basal conditions. This production could be stimulated largely with low concentrations of LH. Another population consisted of larger cells which showed a high basal production but showed hardly any response to LH. A similar situation was described by Walker et al. (1980) for the mammotrophs in the pituitary. They observed a subpopulation of cells which did not respond to TRH and showed a continuous release of prolactin. In addition, a subpopulation of cells was present with stored prolactin which could be stimulated to secrete prolactin under the influence of TRH. Furthermore, there are indications that the population of gonadotrophs in the pituitary is also heterogeneous since different subpopulations showed a difference in the proportional amounts of released LH and FSH (Denef et al., 1980).

I.5.2. Heterogeneity of Leydig cells

A heterogeneity within the population of Leydig cells has been reported by several authors. For example, in the pig the population of Leydig cells can be divided in two subpopulations based on their localization, viz. the "peritubular" Leydig cells (those surrounding one individual tubule) and the "intertubular" Leydig cells (located between different tubules). Van Straaten & Wensing (1978) have demonstrated that

these populations differ profoundly with respect to the activity of various steroidogenic enzymes. There are also indications that the population of Leydig cells in the rat is heterogeneous, but less pronounced than in the pig. A large variation in the degree of staining for 3β-HSD activity and in the number of LH receptors per Leydig cell has been demonstrated (Purvis et al., 1978[b]; Clausen et al., 1981). Subpopulations of Leydig cells, with different density, have been isolated, which showed the same amount of LH receptors, but which were clearly different in LH stimulated testosterone production (Payne et al., 1980) and in the activities of key steroidogenic enzymes (O'Shaugnessey et al., 1981).

It is known that Leydig cells exposed to high amounts of LH or hCG show a decrease in the number of LH receptors and in steroid production and are temporary much less sensitive to hormonal stimulation (Sharpe, 1976; Haour & Saez, 1977; Hsueh et al., 1977; Purvis et al., 1977). Hutaniemi & Martikainen (1978) and Guillou et al. (1985) showed that this desensitization occurs already with physiological levels of LH. It appears possible, therefore, that also *in vivo* Leydig cells may differ in their sensitivity to LH, which may be a cause for heterogeneity within the population of Leydig cells.

Chase & Payne (1983) found a shift of Leydig cells from the low density population to the high density population during sexual maturation, whereas Clausen et al. (1981) observed an increase in the number of LH receptors per cell during puberty. These data suggest that a difference in the stage of differentiation can partly account for the heterogeneity of Leydig cells.

Furthermore, Bergh et al. (1982) have demonstrated that in mature rats perivascular Leydig cells are smaller than peritubular Leydig cells and that in addition the size of the peritubular Leydig cells is correlated with the stage of spermatogenesis in the adjacent tubules, being largest in stages VII-VIII. These results suggest that a difference in localization can also contribute to the heterogeneity of the

Leydig cell population.

There are some similarities between Leydig cells and macrophages (see Chapter I.4.) and there are indications that macrophages may play a role in steroid production. It seems possible therefore, that macrophages can modulate or interfere with Leydig cell functions. If Leydig cells are in contact with different numbers of macrophages this may also contribute to the heterogeneity within the Leydig cell population.

I.6. CONCLUSION

There are many indications that not all Leydig cells have the same properties. This may be caused by e.g. differences in localization, differentiation or sensitivity to LH. This implies that the isolated population of Leydig cells may be heterogeneous with respect to certain functions. In the present study we have investigated different aspects of this heterogeneity in the rat.

CHAPTER II
HETEROGENEITY OF LEYDIG CELLS

II.1. INTRODUCTION

There are indications that the population of Leydig cells is heterogeneous e.g. with respect to the amount of LH-receptors, 3β-HSD activity and the sensitivity to LH (see Chapter I.5.2.). Hence, studies performed with a mixed population of Leydig cells may have resulted in a model for the regulation of steroid production which does not apply to all individual Leydig cells. Fig. II.1 illustrates the possible consequences of studying the hormone responsiveness in a population consisting of 2 cell types, differing in basal activities and sensitivity to hormonal stimulation in a theoretical model. The mixed population shows an activity which is the mean of the activity of the contributing subpopulations. In this example this has led to an underestimation of the stimulatory effect of the hormone. The reality of such a model has been demonstrated for some cell populations, viz. for luteal cells and for mammotrophs (see Chapter I.5.1.).

We have tried to improve the procedure for the isolation of Leydig cells in order to get a higher cell yield and we have attempted to separate different subpopulations of Leydig cells.

II.2. HETEROGENEITY: FACT OR ARTIFACT?

Using morphometric studies Mori & Christensen (1980) have calculated that the number of Leydig cells present in a testis of a mature rat is approximately 30×10^6. It appeared from previous studies that the recovery of Leydig cells in our hands was very low. Moreover, a large variation in LH-stimulated testosterone production (ranging from 3 to 100 ng/10^6 Leydig cells/h, has been reported (Janszen et al., 1976; Grotjan et al., 1978; Cigorraga et al., 1980; Payne et al., 1980; Purvis et al., 1980; Chen et al., 1981; Cooke et al., 1981; Sharpe &

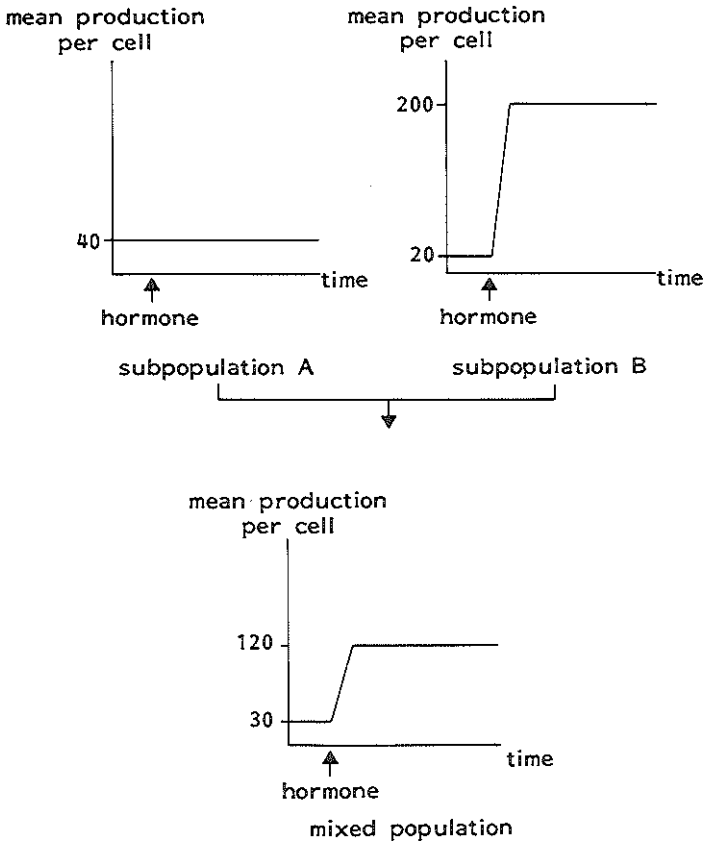


Fig. II.1. Activities of a mixed (heterogeneous) population compared to those of the separate (homogenous) subpopulations (theoretical model).
Subpopulation A (50% of the cells) shows a relative basal activity of 40 and cannot be stimulated by the hormone. Subpopulation B shows a relative basal activity of 20 and can be stimulated 10-fold by the hormone. The basal activity of the 1:1 mixed population will be 30 and the population can be stimulated only 4-fold.

Cooper, 1982). This may partly be due to damage of the cells during the isolation procedure.

We have evaluated, therefore, different isolation procedures with respect to the yield and quality of the isolated cells.

II.2.1. Comparison of different isolation procedures

We have compared three different procedures: collagenase treatment without mechanical forces, collagenase treatment in combination with a gentle mechanical treatment (shaking at 80 cycles/min), which is the standard procedure, and collagenase treatment in combination with a rather rough mechanical treatmen (shaking at 1500 cycles/min). Esterase activity, which can be used as a marker for interstitial cells (Rommerts et al., 1973; van der Vusse et al., 1973) was determined to trace the interstitial cells in various fractions obtained during the isolation. The results, shown in appendix paper I, have led to the following conclusions:

1) mechanical treatment was required to release interstitial cells from the tissue.
2) more esterase activity (representing interstitial cells) was released by shaking at 1500 cycles/min than by the standard method.
3) a large proportion of the released esterase activity was present in the cell-free supernatant, indicating that many cells were disrupted during isolation.
4) the percentage disrupted cells was much higher using the rough method than using the standard method.
5) the esterase activity recovered in the final cell preparation was only approximately 10% and 6% for the standard and the rough method, respectively.

Based on these results we have concluded that we have been unsuccessful to improve the isolation procedure with respect to the yield of cells. Moreover, many cells were severely damaged during the isolation, which suggests that also in the final

cell preparation damaged cells may be present.

II.2.2. Quality of Leydig cell preparations

To investigate the possible presence of damaged cells, we have studied the quality of cell preparations obtained with two methods, described in Chapter II.2.1., by measuring the effect of LH and NADPH on testosterone production. Stimulation of testosterone production by LH requires intact cells, whereas NADPH can only stimulate cells with damaged membranes (Tsang & Stachenko, 1970; Goverde et al., 1980). We found that cells obtained by the rough method (shaking at 1500 cycles/min) did not respond to LH, but could be stimulated by NADPH. Cells obtained by the standard method (shaking at 80 cycles/min) could be stimulated by LH and also slightly by NADPH. Hence, cell preparations obtained by the rough method contained no or a very low number of intact cells and many damaged cells, whereas cells obtained by the standard method contained both damaged and intact cells. As is shown in appendix paper I, we were able to improve the quality of the cell preparation by selection of the cells which attached to plastic. This was demonstrated by an increase in the ratio stimulation factor by LH: stimulation factor by NADPH. In addition we have used a histochemical viability test (see appendix paper I), which demonstrated that the percentage damaged cells after attachment had decreased from approximately 27% to approximately 2%. However, there were still some damaged cells in the final preparation. The presence of these damaged cells results in a Leydig cell population which is heterogeneous with respect to steroidogenic activities. This heterogeneity is the result of an isolation artifact.

The separation of subpopulations of Leydig cells differing in steroidogenic capacities as described by Cooke et al. (1981) and Payne et al. (1980) is based on differences in cell density. These authors have used procedures for the isolation of Leydig cells which are similar to our standard procedure and

it is likely, therefore, that these preparations also contained damaged cells. Moreover, observations from Janszen et al. (1976) suggest that cell damage can lead to changes in density and Laws et al. (1985) have reported the presence of Leydig cell fragments in a low density population. It seems likely, therefore, that the "Leydig cells" present in the low density region represent, at least partly, damaged Leydig cells. Thus, it is most likely that the isolation procedure has also contributed to the heterogeneity observed by the authors mentioned above. Since the percentage damaged cells in the preparations may vary, this can also account for the large variation in steroid production which has been reported.

II.3. THE CELL SORTER: A TOOL FOR SEPARATION OF LEYDIG CELLS?

In addition to our studies dealing with the recovery and quality of Leydig cells (see Chapter II.2.) we have investigated the possibility to separate subpopulations of Leydig cells with different properties. Many investigators have separated functionally different Leydig cells based on differences in cell density. However, the presence of damaged cells (see Chapter II.2.) limits the usefulness of this method. We have tried, therefore, to separate cells using a Fluorescent Activated Cell Sorter (FACS) after labelling with specific fluorescent probes.

This method has the following advantages: 1) cells can be labelled based on a variety of properties, 2) the intensity of the fluorescence is analyzed quantitatively and 3) each cell is detected and can be isolated individually. This procedure has the disadvantage that it is rather time consuming which is a limitation when large amounts of cells are required for biochemical studies.

The following properties of Leydig cells seemed appropriate to investigate for separation of subpopulations: the number of LH receptors (Purvis et al., 1978[b]; Clausen et

al., 1981), LHRH receptors (Sharpe & Fraser, 1980), the amount of smooth endoplasmic reticulum and the variations in cell size (Bergh, 1982).

Although the FACS seemed a useful means for the separation of different subpopulations of Leydig cells, we expected to meet some practical problems. For example, our preparations contained damaged cells (see Chapter II.2.), which can be a source of error in receptor labelling studies, since these cells do not only bind the ligand to cell surface receptors, but also take up the molecule into the cell (Bohn, 1980). In addition, labelling of the LH and LHRH receptors will result in a stimulation of the steroid production, which may interfere with later studies dealing with regulation of steroid production. Furthermore, separation of cells based on differences in cell size may be hindered by the presence of cell fragments and damaged cells.

Yet we have performed some pilot experiments, in which we have tried to obtain different subpopulations of Leydig cells from mature rats.

LH and LHRH receptors

Labelling of LH and LHRH receptors was performed essentially as described by Purvis et al. (1978[a]) and Hazum et al. (1980), respectively. Before using the FACS, binding was checked by means of fluorescence microscopy. In both cases no fluorescence was observed. The lack of visible fluorescence may have resulted from a poor technique for labelling or from a weak fluorescence which was below the detection limit. Because of this negative result in combination with the disadvantages of this method mentioned above, we have discarded the idea to use membrane receptors for the separation of different cell populations.

Cell size

For separation of Leydig cells based on differences in cell size we have analyzed the preparation with respect to the "scatter" signal (in general it is true that the scatter signal increases with increasing cell size). We observed only a single population. Attempts to collect a population of "small cells" (low scatter signal) and a population of "large cells" (high scatter signal) were unsuccesful because of the presence of cell fragments (low scatter) and cell clusters (high scatter). Moreover, cell clusters may contain cells of various sizes.

Smooth endoplasmic reticulum

Cells were labelled with the carbocyanine dye, 3,3'-dihexyl-2,2'-oxacarbocyanine, which stains specifically the endoplasmic reticulum (Habicht & Brune, 1980). A bright fluorescence was observed. However, it appeared that incubation of the cells with this compound resulted in a 3-fold increase of the basal steroid production and a 2-fold decrease of the LH stimulated steroid production, which makes this dye unsuitable for our studies.

The results which we obtained and the knowledge from our previous studies that the Leydig cell preparations always contain damaged cells made it unattractive to continue our attempts to isolate different subpopulations of Leydig cells by cell sorting.

II.4. CONCLUSIONS

Leydig cell preparations obtained from testes of mature rats always contain damaged cells which results in an artificial heterogeneity of the Leydig cell population *in vitro*. Moreover, the recovery of Leydig cells after isolation is very low (approximately 10%), which implies that the

isolated Leydig cell population may not be representative for the Leydig cell population *in vivo*.

Furthermore we have been unable to separate different subpopulations of Leydig cells, which may be explained partly by the poor quality of the cell preparation and partly by the (unsuitable) parameter which was used for fluorescent labelling.

CHAPTER III
CHARACTERISTICS OF LEYDIG CELLS AND TESTICULAR MACROPHAGES

III.1. INTRODUCTION

The interstitial tissue of the adult rat testis contains in addition to Leydig cells approximately 15-20% macrophages (Dym & Madwha Raij, 1977; Ash & Parker, 1978; Miller, 1982). These macrophages appear similar to those present in other tissues, since they contain lysosomes, coated vesicles, F_c-receptors and numerous surface projections, are active in phagocytosis and show a high acid phosphatase and non specific esterase activity (Miller et al., 1983; Yee & Hutson, 1983), but little is known about their function. Striking is the close association and contact of testicular macrophages with the Leydig cells in the interstitium (Connell & Christensen, 1975; Wing & Lin, 1977; Miller et al., 1983; Bergh, 1985). Moreover, Bergh (1985) has shown that the mass and size of both Leydig cells and macrophages were reduced after unilateral cryptorchidism, whereas Wing & Lin (1977) have demonstrated in the golden hamster that the cell size of both Leydig cells and macrophages diminishes and that there is a reduction of cytoplasmic differentiation during the period that the testis is inactive in germ cell production. These data suggest a possible interaction between Leydig cells and macrophages in the testis. There are also indications that macrophages and steroid producing cells may have common properties. For example, adrenal macrophages show a high 17α-hydroxylase activity (Vernon-Roberts, 1969), whereas 3β-Hydroxy Steroid Dehydrogenase, 3α-Hydroxy Steroid Dehydrogenase, 17β-Hydroxy Steroid Dehydrogenase and 5α-reductase were observed in peritoneal (Milewich et al., 1982) and alveolar macrophages (Milewich et al., 1983; Lofthus et al., 1984). Furthermore Vernon-Roberts (1969) reported that ovariectomy resulted in the reduction of the number of macrophages in the endometrium and in an increase in the level of blood cholesterol, both of which

could be normalized by injection of oestrogens. This may indicate that macrophages play a role in cholesterol transport to steroid producing cells. Kirsch et al. (1981) demonstrated that peritoneal macrophages in coculture with granulosa cells stimulated the progesterone production by these cells.

On the other hand Leydig cells show some properties which are generally ascribed to macrophages. They have the capacity to bind lymphocytes specifically (Born & Wekerle, 1981; Rivenson et al., 1981). There are indications that they are active in the production of prostaglandins (Haour et al., 1979; Molcho et al., 1984) and they show a high non-specific esterase activity (Schnelle et al., 1983).

We have compared some properties of Leydig cells and testicular macrophages in isolated cell preparations to evaluate to which extent Leydig cells and macrophages have common properties.

III.2. COMPARISON OF PROPERTIES OF LEYDIG CELLS AND MACROPHAGES

III.2.1. Pregnenolone production

We have studied the possibility that testicular macrophages may produce steroids by measuring pregnenolone production in vitro. Inhibitors of 17α-hydroxylase and 3β-Hydroxy Steroid Dehydrogenase were added to inhibit pregnenolone conversion. The hormone responsiveness was studied by incubation of cells in the presence of LH and the cholesterol side chain cleavage (CSCC) activity was investigated by studying the effect of 22-R-OH-cholesterol, a substrate for the CSCC enzyme complex. The activity of testicular macrophages was compared with the activities of Leydig cells and peritoneal macrophages. From the results given in Table III.1. we have concluded that, in contrast to Leydig cells, both testicular and peritoneal macrophages are not active in pregnenolone production and do not respond to LH. Furthermore, the activity of the CSCC enzyme of the macrophages

was insignificant compared to the activity of the Leydig cells.

Table III.1. Pregnenolone production by Leydig cells, testicular macrophages and peritoneal macrophages.

	pMol pregnenolone/h/3x10^5 cells		
preparation	basal	LH (100 ng/ml)	22R-OH-chol. (19 µM)
Leydig cells	36.0 ± 5.1*	120.3 ± 38.6*	743.6 ± 177.4*
testicular macrophages	1.0 ± 0.1	1.9 ± 0.8	7.1 ± 2.2
peritoneal macrophages	0.7 ± 0.1	0.7 ± 0.1	2.0 ± 0.9

*significantly different compared to macrophage preparations ($p<0.05$)

Cell preparations were obtained and characterized as described in appendix paper II. 3x10^5 cells were preincubated for 90 min and subsequently incubated for 60 min in the presence of inhibitors of pregnenolone metabolism. Pregnenolone was measured in the media by radioimmunoassay, according to Rommerts et al. (1982).
Means ± SD of 4 incubations of 2 different cell preparations.

III.2.2. 17α-Hydroxylase activity

The results in Table III.1. show that it is unlikely that testicular macrophages contribute significantly to the pregnenolone production by the testis. Vernon-Roberts (1969) reported that the macrophages in the adrenal gland contribute to a large extent to the 17α-hydroxylation. It seems possible, therefore, that macrophages are involved in the conversion of pregnenolone.

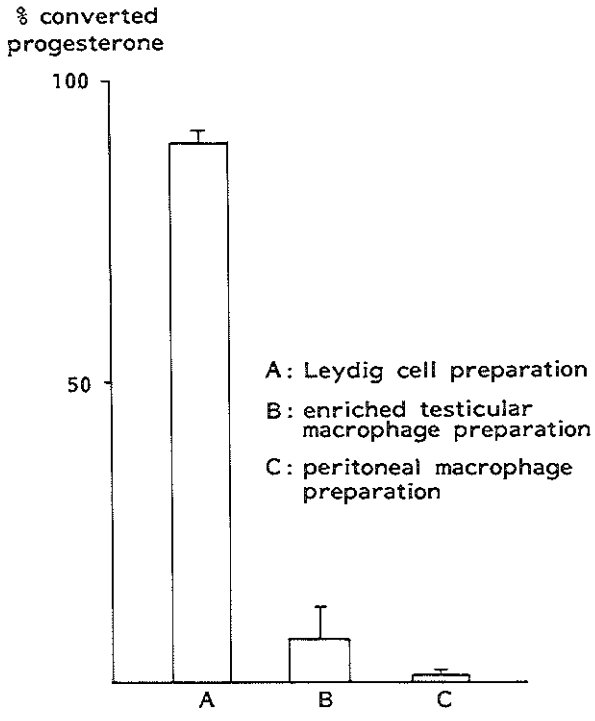


Fig. III.1. 17α-hydroxylase activity in Leydig cells, testicular macrophages and peritoneal macrophages. Preparations were obtained as described in the legend of Table III.1. 10^6 cells were incubated in 2 ml medium containing 100 ng progesterone and ^{3}H-progesterone (approximately 10^6 dpm) for 1 h. Steroids were extracted from the medium by ethyl acetate and separated by thin layer chromatography. (toluene: ethyl acetate = 3:2 (v/v)). Individual spots of progesterone, 17α-OH-progesterone, androstenedione and testosterone were scraped of the plate and the radioactivity was determined. The percentage of unmetabolized progesterone and the percentage of metabolites (17α-OH-progesterone, androstenedione, testosterone) was calculated.
Means ± range of 2 different cell preparations.

In this respect we have compared the 17α-hydroxylase activity of Leydig cells, testicular macrophages and peritoneal macrophages. The results in Fig. III.1. show that under incubation conditions whereby Leydig cell preparations had converted more than 90% of the radioactive labelled progesterone, less than 10% of this substrate was converted by testicular and peritoneal macrophage preparations. We have concluded, therefore, that the macrophages in the testis hardly contribute to the total testicular 17α-hydroxylation activity.

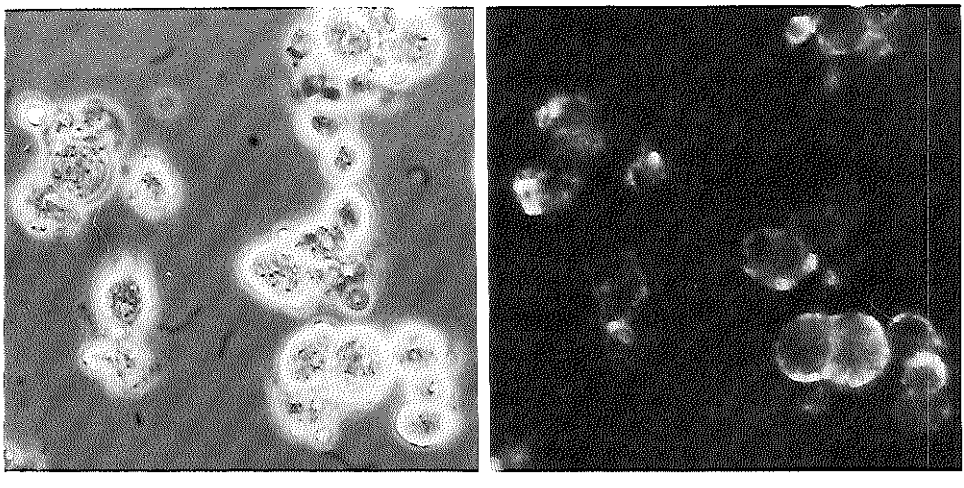
III.2.3. **Non-specific esterase**

Non-specific esterase is generally used as marker enzyme for macrophages (Brade, 1982; v. Furth & Diesselhof-den Dulk, 1982; Onozaki et al., 1983; Yee & Hutson, 1983). In interstitial cell preparations obtained from mature rat testes non-specific esterase activity was correlated with 3β-HSD activity, hCG-binding and LH-stimulated steroid production (van der Vusse et al., 1973; Janszen et al., 1976; Cooke et al., 1981; Kühn-Velten et al., 1982). We have studied this apparent similarity between Leydig cells and macrophages using both a quantitative and a qualitative assay for the detection of esterase activity (see appendix paper II).

It appeared that Leydig cells from mature rats showed an approximately 25-fold higher esterase activity than testicular macrophages. This could also be demonstrated histochemically. Leydig cells exhibit a positive staining already after 30 seconds, whereas testicular macrophages become positive only after 3 minutes. Hence, non-specific esterase activity is not only a characteristic which Leydig cells and macrophages have in common, but it is even much more pronounced in Leydig cells. Since the specific function of this particular enzyme is unknown, the relevance of this observation is still not clear.

III.2.4. Macrophage membrane-specific antigens

Macrophages as well as Leydig cells have the capacity to bind lymphocytes to their surface. Since it seems likely that specific membrane properties are responsible for this selective binding, we have investigated whether macrophages and Leydig cells have certain membrane antigens in common using various monoclonal antibodies raised against macrophage membrane antigens. We have found that one antibody, which is characterized by its selective binding to macrophages in the lymphoid organs and the liver (Rozing, personal communication) was bound by more than 90% of the cells in a standard Leydig cell preparation from mature rats (Fig. III.2.). No binding was observed in preparations from immature rats, indicating the



phasecontrast fluorescence

Fig. III.2. Leydig cell preparation labelled with antibodies against macrophage membrane specific antigens.
A Leydig cell preparation was obtained using the standard isolation procedure described in appendix paper I and the cells were allowed to attach to the plastic petri dishes. After removal of the floating cells, the attached cells were incubated successively with mouse α-rat macrophage membrane antigen, rabbit α-mouse (F_{ab} fragments) and goat α-rabbit-FITC.

specificity of the binding to mature cells. Furthermore, binding of total rabbit IgG, but not of F_{ab}-fragments, was observed (not shown), suggesting that F_c-receptors are present on Leydig cells. These data indicate the presence of at least two membrane antigens, which are considered to be specific for macrophages, on the membrane of Leydig cells from mature but not from immature rats. Since the antigen which binds the monoclonal antibody has not been identified, the function of this antigen on the Leydig cell membrane is not clear. The possible presence of F_c-receptors, however, may explain the specific binding of lymphocytes, since these cells often bear IgG's on their surfaces.

III.2.5. Prostaglandin E_2 production

In addition to their phagocytic activity macrophages are active in the secretion of many biologically active substances such as enzymes, enzyme inhibitors, plasma proteins, mitogens, lipids, etc. (for reviews see Werb (1983) and Takemura & Werb (1984)). Prostaglandin E_2 (PGE_2) is one of these biologically active lipids (Davies & Bonney, 1979; Hsueh et al., 1979; Stenson & Parker, 1980; Hume & Gordon, 1983). Haour et al. (1979) have demonstrated that Leydig cell preparations produce PGE_2 and that this production could be stimulated approximately 5-fold after *in vivo* pretreatment with hCG. Molcho et al. (1984) found that LHRH could stimulate the PGE_2 production *in vitro*. These data suggest that Leydig cells may be involved in PGE_2 production. We have extended these experiments by comparing the testicular PGE_2 production *in vivo* of normal rats with the production of rats with testes which were depleted of Leydig cells but still contained macrophages (LCD rats) (see *appendix paper III*).

As is shown in Table III.3., the basal concentration PGE_2 was slightly elevated in LCD testes when compared with normal testes. Treatment with hCG resulted in an approximately 300-fold increase in PGE_2 concentration in normal testes whereas

only an approximately 6-fold increase was observed in LCD testes. This clearly shows that Leydig cells are involved in the PGE_2 production in the testis. Whether the Leydig cells are the actual source of the prostaglandins is not known. One possibility is that hCG directly stimulates the PGE_2 production by Leydig cells, but another possibility is that other cells, probably the macrophages, produce PGE_2 at a basal rate and are stimulated via an interaction with the Leydig cells. If macrophages contribute to the prostaglandin E_2 production in the testis, an influx of macrophages in the LCD testis as a result of the destruction of Leydig cells (which is suggested by Kerr et al. (1985[a]) and Morris (1986)) might account for the elevated basal concentration when compared to the normal testis. Remarkable is the stimulatory effect of hCG in the LCD rat. We have observed that after depletion of the Leydig cells LH-sensitive Leydig cell precursors remain in the testes (see appendix paper IV). These cells, being the target for hCG, may be responsible for the increased PGE_2 levels in LCD testes after treatment with hCG.

Table III.3. Prostaglandin E_2 concentration in testis tissue from normal and from Leydig Cell Depleted (LCD) adult rats.

	treatment	ng PGE_2/testis
normal rats	control	2.3 ± 0.6 (3)
	hCG	608.3 ± 68.3 (3)+
LCD rats	control	3.8 ± 0.9 (3)*
	hCG	21.8 ± 13.8 (3)+

* significantly elevated compared to normal rats ($p<0.05$)
\+ significantly elevated compared to control ($p<0.005$)

LCD rats were obtained as described in appendix paper III. Rats were pretreated in vivo with hCG (100 i.u., subcutaneously) or saline (control). Testes were removed 6 hours later, decapsulated and sonicated (at 0°C). PGE_2 concentrations were determined in the sonicate by radioimmunoassay.
Means ± SD, n=3.

III.3. CONCLUSIONS

Our results show that Leydig cells and macrophages have many different characteristics. Leydig cells were active in steroid production, as was reflected by the presence of 3β-HSD activity, 17α-hydroxylase activity and LH-stimulated pregnenolone production, in contrast to testicular macrophages which showed none of these activities. Non-specific esterase, which is generally used as a marker for macrophages, was much more active in Leydig cells and is, therefore, in the testis a more specific marker for Leydig cells than for macrophages. Hence, Leydig cells and macrophages show clearly different properties.

On the other hand we have found that Leydig cells and macrophages show some similarities with respect to their membrane antigens. Particularly the presence of F_c-receptors may account for the selective binding of lymphocytes, which is observed for both macrophages and Leydig cells.

Furthermore we have found that the production of prostaglandins E_2 in the testis is under the influence of LH and depends on the presence of Leydig cells. Whether the Leydig cells are the actual source of PGE_2 production or only the mediators involved in the production has not yet been elucidated.

CHAPTER IV
DESTRUCTION AND REGENERATION OF LEYDIG CELLS IN MATURE RATS

IV.1. INTRODUCTION

After the regression of the foetal population of Leydig cells soon after birth, differentiation of Leydig cells starts again during the onset of puberty, resulting in the adult population of Leydig cells. This differentiation takes place during sexual maturation, and is dependent on both LH and FSH (Odell et al., 1973; van Beurden et al., 1976; Chen et al., 1976; Kerr & Sharpe, 1985[a,b]). It is not clear whether this differentiation continues in testes after maturation, since little is known about the turnover of Leydig cells. Christensen & Peacock (1980) have demonstrated that hCG treatment of mature rats resulted within 5 weeks in a 3-fold increase in the number of Leydig cells. This implies that after maturation the testis is still capable to generate new Leydig cells. This hyperplasia following gonadotrophic stimulation could be the result of either differentiation of precursor cells, comparable to the differentiation during normal sexual development, or mitosis of existing Leydig cells as suggested by Kerr et al. (1979).

Administration of the compound Ethylene Dimethane Sulfonate (EDS) to mature rats causes a decrease in androgen levels, with a resulting loss in weight of the ventral prostate, seminal vesicles and epididymis, and disruption of spermatogenesis (Jackson & Morris, 1977; Morris & McCluckie, 1979; Jackson & Jackson, 1984). The effects of EDS on spermatogenesis and the accessory organs could be prevented by simultaneous administration of testosterone (Jackson & Jackson, 1984), which suggests that EDS primarily injures the Leydig cells. We have found that EDS actually destroys the Leydig cells (see Chapter IV.2.)

Studies from Jackson & Morris (1977) and Morris & McCluckie (1979) have shown that the effect of EDS on testosterone production and the accessory organs is temporary.

Because the Leydig cells are destroyed after EDS treatment, this implies that repopulation of Leydig cells must have occurred in these mature rats. We have investigated the repopulation of Leydig cells following EDS treatment in normal mature rats and in sterile (germ cell depleted) mature rats, where paracrine regulation may be different because of an altered Sertoli cell function (Rich & De Kretser, 1977). We have investigated also the possible role of LH and FSH in this repopulation (see Chapter IV.3.).

IV.2. SELECTIVE DESTRUCTION OF LEYDIG CELLS BY ETHYLENE DIMETHANE SULFONATE (EDS)

Administration of diesters of methane sulphonic acid (general formula: $CH_3-OSO_2-(CH_2)_n-OSO_2-CH_3$) causes sterility in the male rat. Methylene Dimethane Sulphonate (MDS; n=1), Propylene Dimethane Sulphonate (PDS; n=3) and Butylene Dimethane Sulphonate (busulfan) (BDS; n=4) specifically inhibit the normal development of spermatogonia. As is shown in appendix paper III, Ethylene Dimethane Sulfonate (EDS, n=2) acts on the Leydig cells. Leydig cells from normal mature rats are selectively destroyed 72 h after administration of EDS, resulting in a decrease of blood testosterone to castration levels. Macrophages in the interstitium, however, remained intact. The appearance of the spermatogenic epithelium was still normal after 72 h, but thereafter spermatogenesis was disturbed probably due to a lack of testosterone.

We have also studied the effect of administration of EDS to sterile mature rats (prenatally irradiated). In the testes of these rats germ cells are absent, but Sertoli cells and Leydig cells are present and appear microscopically normal. 72 H after administration of EDS to these rats, the number of Leydig cells in the testes and the concentration of testosterone in the blood were extremely low, but a few Leydig cells were still present as was seen in histological sections (see appendix paper IV).

Administration of EDS to immature rats did not affect the steroid production by isolated interstitial cells, which indicates that Leydig cells from immature rats are resistant to EDS (see appendix paper III).

From the results of appendix papers III and IV it was concluded that administration of EDS to normal mature rats results in a Leydig Cell Depleted (LCD) testis, whereas administration to sterile (prenatally irradiated) mature rats resulted in a testis which contains only a very few Leydig cells. These two experimental situations have been used to study the development of Leydig cells after sexual maturation (see Chapter IV.3.).

IV.3. REPOPULATION OF LEYDIG CELLS IN MATURE RATS AFTER TREATMENT WITH ETHYLENE DIMETHANE SULFONATE (EDS)

EDS was administered to normal mature rats to obtain Leydig Cell Depleted (LCD) rats (see Chapter IV.2.). We have used these rats to study whether in mature rats, in the absence of Leydig cells, differentiation of new Leydig cells can occur. Furthermore we have studied a possible role of the gonadotrophic hormones on the repopulation of Leydig cells. The results, shown in appendix paper IV, show that repopulation of functionally active Leydig cells occurs within approximately 5 weeks after EDS administration. Since Clegg and McMillan (1966) have suggested that testicular macrophages can transform into cells with Leydig cell characteristics and since we observed that the macrophages remain in the testis after EDS treatment, we have investigated the possibility that these macrophages might give rise to the new population of Leydig cells. In this respect macrophages were labelled by intraperitoneal injection with trypan blue or intratesticular injection with fluorescent beads 48 h before the EDS treatment. 5 Weeks after administration of EDS interstitial cells were isolated from the testes. Cells labelled with trypan blue or fluorescent beads could be identified in the isolated cell preparation, but these

cells never exhibited 3ß-HSD staining which was characteristic for the newly formed Leydig cells in the preparation. On basis of this result we have rejected the hypothesis that the new population of Leydig cells originates from testicular macrophages.

After EDS treatment of rats with suppressed endogenous LH and of hypophysectomized rats it appeared that the repopulation of Leydig cells was dependent on LH, whereas the presence of other pituitary hormones such as FSH and prolactin was not required (see appendix paper IV). This implies that an LH-dependent precursor cell for Leydig cells remains in the testis after EDS treatment.

Administration of EDS to sterile (prenatally irradiated) mature rats also resulted in an enormous destruction of Leydig cells, but a few Leydig cells remained in the testis. The restoration of Leydig cells and of steroidogenic activities in sterile rats after treatment with EDS was much faster than in EDS treated normal rats. This difference in the rate of repopulation might have been influenced by e.g. 1) the presence of some Leydig cells which survived the treatment and which may have led to a faster repopulation; 2) the local environment of the interstitial tissue (altered Sertoli cell function) in sterile rats which might favour a faster repopulation of Leydig cells; 3) the relatively high levels of gonadotrophins in sterile rats already before the EDS treatment which might have increased the number of precursor cells and 4) the higher levels of gonadotrophins in sterile rats compared to normal rats soon after EDS treatment, particularly of LH which may be the trigger for Leydig cell differentiation, and which might have caused an earlier onset of Leydig cell repopulation.

IV.4. CONCLUSIONS

We have found that EDS specifically destroys Leydig cells from mature rats, but not from immature rats. In sterile (prenatally irradiated) mature rats the majority of the Leydig

cells is destroyed, but a few Leydig cells survived the treatment.

Repopulation of Leydig cells after EDS treatment occurs and is faster in sterile than in normal rats. This repopulation is dependent on LH and not on FSH, in contrast to the development of Leydig cells during normal sexual maturation, where both hormones are required.

CHAPTER V
DEVELOPMENTAL CHANGES IN THE INTERSTITIUM

V.1. INTRODUCTION

During sexual development the testes show significant changes. The most remarkable changes take place in the tubular compartment, where the maturation of germ cells has been initiated, which leads to an enormous increase in weight. At the same time the interstitium also undergoes many changes. The number of Leydig cells increases and concomitantly the composition of the various cell types in the interstitium changes. The Leydig cells show a change in several properties such as an increase in size and the number of LH-receptors and altered pathways for pregnenolone metabolism.

We have characterized the composition of the interstitial cell preparations from immature and mature rats using morphological and functional parameters (see Chapter V.2.) and we have studied the change in steroidogenic properties of Leydig cells during maturation (see Chapter V.3.).

V.2. COMPOSITION OF THE INTERSTITIUM BEFORE AND AFTER MATURATION

In mature rats the Leydig cells form the vast majority of the cells present in the interstitium and macrophages contribute for approximately 15-20% (Christensen, 1975; Dym & Madhwa Raj, 1977; Ash & Parker, 1978; Miller, 1982). Kerr & Sharpe (1985[b]) have reported that in immature rats the number of Leydig cells was approximately equal to the number of mesenchymal cells. As far as we know there are no clear data about the occurrence of macrophages in the interstitium of immature rats.

In our studies we have used mainly isolated Leydig cells. For the isolation interstitial cells were released from the testes, purified, and the percentage Leydig cells was estimated

by staining for 3β-HSD activity (marker for Leydig cells). Based on this marker interstitial cell preparations from mature rats contain approximately 80% Leydig cells and from immature rats approximately 43% (see Table V.II). In several previous studies we have calculated the steroid production per Leydig cell by estimation of the percentage 3β-HSD positive cells in the preparation. However, the identity of the cells showing no 3β-HSD activity is unknown and the possibility that these cells are also involved in steroid production cannot be excluded a priori. It seemed useful, therefore, to characterize the unknown fraction in the preparations.

We have studied the possibility that the unknown cell fraction contained macrophages. Therefore the macrophages in the preparations have been labelled in vivo with trypan blue (for method see appendix paper III). It appeared that preparations from immature rats (21-23 days old) contained less than 1% (n=4) trypan blue containing cells, whereas in preparations from mature rats 20 ± 6% (n=4) trypan blue containing cells were found. The percentage found in the preparation from mature rats fits in with the percentages reported by other authors mentioned above. Hence, the preparation from mature rats contain almost exclusively Leydig cells (approximately 80%) and macrophages (approximately 20%). As is described in Chapter III macrophages do not contribute significantly to the steroid production in vitro. The presence of these cells in the preparation, therefore, will probably not lead to an overestimation of the steroid production per Leydig cell when based on the percentage 3β-HSD positive cells in the preparation. In contrast, the very low number of trypan blue containing cells in preparations from immature rats, indicates that the occurrence of macrophages in these rats is very low. The possibility that the interstitium from immature rats contain macrophages which are not active in phagocytosis can not be excluded yet. Therefore we have characterized the isolated interstitial cell preparation from immature rats also morphologically and compared the cells with those present

in situ in testes from immature and mature rats.

Macrophages, which can be characterized by their pale staining cytoplasm containing large vacuoles and an irregular pale staining nucleus with clumped peripheral heterochromatin (Kerr & Sharpe, 1985[b]; Laws, 1985) were clearly present in the interstitial tissue from testes of mature rats (Fig. V.1[a]), but were almost absent in the interstitium from testes of immature rats (Fig. V.1[b]) and in interstitial cell preparations isolated from testes of immature rats (Fig. V.1[c]). This observation supports the conclusion that interstitial cell preparation isolated from testes of immature rats hardly contain macrophages and it indicates that the percentage macrophages present in situ is also very low. Leydig cells, which can be characterized by their round or oval, dark staining nucleus, containing prominent nucleoli and a thin rim of peripheral heterochromatine, and a dark staining cytoplasm (Kerr & Sharpe, 1985[a,b]; Laws, 1985) are clearly present in the interstitium of testes from mature rats (Fig. V.1[a]) and from immature rats (Fig. V.1[b]) and in isolated interstitial cells from immature rats (Fig. V.1[c]). In the interstitium from testes of immature rats we observed also "peritubular" cells (Fig. V.1[b]) which can be identified by their elongated shape, flattened discoid-like nucleus and very little accompanying cytoplasm. Laws et al. (1985) have reported that these cells round up during the isolation procedure, resulting in cells showing many digitating processes and containing an eccentric crescent-shaped nucleus and suggested that these cells represented mesenchymal cells. Similar cells were frequently seen in the isolated cell preparation from immature rats (Fig. V.1[c] and V.2[b]). Since the tubules are surrounded by a layer of endothelial cells we have considered the possibility that these cells may also form part of the "unknown" cell fraction. We have performed some pilot studies. Staining for alkaline phosphatase and binding of acetyl LDL (low density lipoprotein) (both characteristics of endothelial cells) showed only a very few positive cells. It seems likely, therefore, that the fraction of peritubular cells

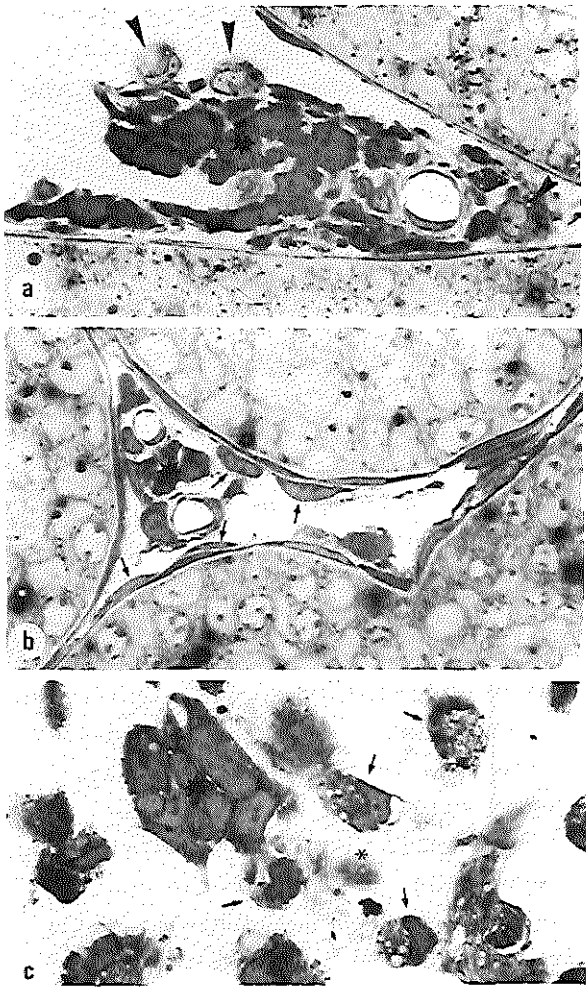


Fig. V.1.:

a. cross section of a mature (60-90-day-old) rat testis. x 720.
✸ cluster of Leydig cells
➤ macrophages

b. cross section of an immature (22-day-old) rat testis. x720.
✸ cluster of Leydig cells
→ peritubular cells

c. cross section from interstitial cell preparation from immature (22-day-old) rat testes. x720.
✸ cluster of Leydig cells
→ mesenchymal cells
* undefined cells

Testes (a,b) were fixed by perfusion fixation with a solution of 2% glutaraldehyde, 3% formaldehyde in 0.1 M phosphate buffer, pH 7.3 with 3% polyvinyl pyrrolidon according to Forssmann et al. (1977). Cells (c) were fixed in the same solution after attachment for 1 h. Testes and cells were embedded in Epon. 1 µm sections were cut and stained with toluidine blue.

which is present in the isolated cell preparation mainly contains mesenchymal cells.

The proportions of Leydig cells and mesenchymal cells in a preparation from immature rats have been counted (see Table V.1.). The preparation contained mainly Leydig cells (62%), whereas 34% consisted of mesenchymal cells. A small number of cells (4%), called "undefined" cells, did neither show the characteristics of Leydig cells nor of mesenchymal cells. No macrophages were seen in the counted areas.

Table V.I. Composition of interstitial cell preparation obtained from testes of immature rats (22 days old).

cell type	percentage
Leydig cell	62
Mesenchymal cell	34
Undefined cell	4

232 cells were counted in 9 different areas.

Fig. V.2. shows a transmission electron micrograph of a cluster of Leydig cells (a) and a cluster of mesenchymal cells (b). Abundant smooth endoplasmic reticulum is present in the cytoplasm of the Leydig cells (c).

Our morphological data show that this interstitial cell preparation from immature rats contains 62% Leydig cells in contrast to the mean of 43% which was found in other cell preparations using 3β-HSD as marker for Leydig cells (see Chapter V.2.3.). This discrepancy might reflect that during differentiation the precursors of Leydig cells acquire the morphological appearance of Leydig cells before they develop their steroidogenic properties. Furthermore, it has been suggested by Laws (1985) and Kerr & Sharpe (1985[b]) that the mesenchymal cells are the precursor cells for the Leydig cells.

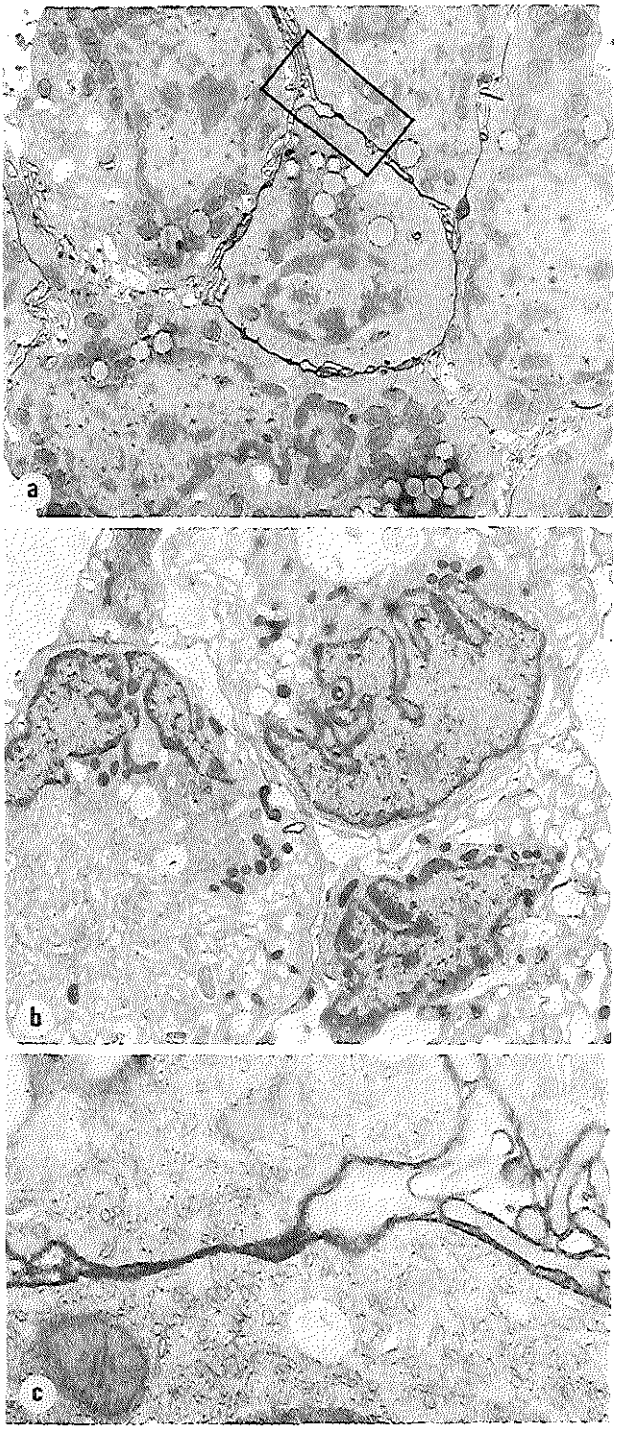

a
b
c

Fig. V.2. Transmission electron micrographs of an isolated interstitial cell preparation from immature (22-day-old) rat testes.
a. cluster of Leydig cells. 3660x.
b. cluster of mesenchymal cells. 3660x.
c. detail (rectangle) from a. 33280x.

Taken together, these data indicate that the interstitial cell preparations from immature rats contain precursors for Leydig cells, partially differentiated Leydig cells and differentiated Leydig cells. Whether the precursor cells and the partially differentiated cells contribute to the steroid production is not known (yet) and separation of the various cell types is required to study this possibility.

V.3. CHANGES IN STEROIDOGENIC PROPERTIES OF LEYDIG CELLS DURING DEVELOPMENT

During testicular development functional properties of Leydig cells change. The size of the Leydig cells (Knorr et al., 1970; Kerr & Sharpe et al., 1985[a]) and the number of LH-receptors per cell (Ketelslegers et al., 1978; Clausen et al., 1981) increase during maturation. Furthermore a change in pathways of pregnenolone metabolism has been observed during maturation (see review van der Molen & Rommerts, 1981). The main steroids produced by immature rats are 5α-reduced metabolites. Testosterone is not an obligatory intermediate in steroid metabolism in immature rats, whereas in mature rats testosterone is the main end product. It has been reported, however, that the total amount of steroids produced per Leydig cell did not change during maturation (Purvis et al., 1978[c]; van der Molen et al., 1980), indicating a constant activity of the cholesterol side chain cleavage enzymes. Our results, shown in appendix paper I, show that freshly isolated Leydig cell preparations obtained from mature rats always contained damaged cells in addition to intact cells and that the selection of the intact cells by means of attachment resulted in a significant

increase in the testosterone production when expressed per Leydig cell. Hence, it seems likely that in previous studies the steroidogenic capacity of Leydig cells from mature rats has been underestimated. To evaluate whether the steroidogenic capacity of the cell changes during maturation, we have compared the steroidogenic activities of Leydig cells in vitro obtained from immature rats and from mature rats after selection of the intact cells in the preparation. In addition we have studied in Leydig cell preparations obtained from rats of different ages two other parameters both reflecting the maturation of Leydig cells, viz. the relative conversion of pregnenolone to testosterone and the non-specific esterase activity (marker enzyme for Leydig cells from mature rats, see appendix paper II).

V.3.1. Steroidogenic capacity

The steroidogenic capacity of Leydig cells from immature (21-23 days old) and from mature (60-90 days old) rats was estimated by measuring pregnenolone production, under basal conditions, after stimulation with either LH or db-cAMP, or in the presence of 22-R-OH-cholesterol (a substrate for the cholesterol side chain cleavage enzymes). The pregnenolone production by Leydig cells from mature rats was under all incubation conditions at least 4-times higher than the production by Leydig cells from immature rats (Fig. V.3.). Hence, the steroidogenic capacity of Leydig cells clearly increases during maturation. Even when the supposed Leydig cell precursors present in the interstitial cell preparations from immature rats (see Chapter V.2.) would contribute to some extent to the steroid production, the steroid production by Leydig cells from mature rats is still significantly higher. The stimulatory effect of dbc-AMP was not different from the effect of LH which indicates that the lower number of LH-receptors, present on Leydig cells from immature rats, is not responsible for the difference in LH-stimulated steroid

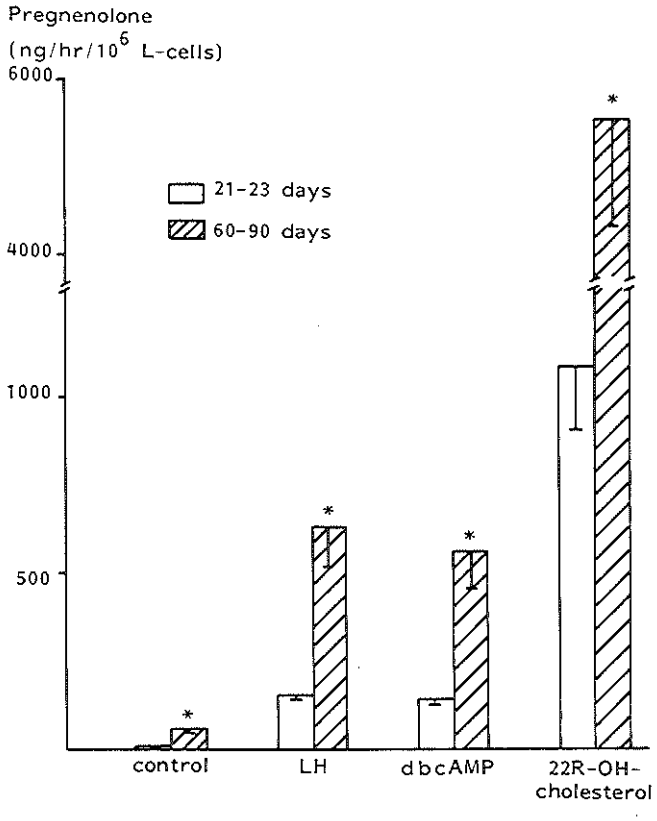


Fig. V.3. Steroidogenic capacity of Leydig cells from 21-23- or 60-90-day-old rats.
Leydig cell preparations were obtained as described in appendix paper I. Cells were incubated for 60 min without additions and subsequently incubated for 60 min in the presence of inhibitors of pregnenolone metabolism. Leydig cells were characterized by staining for 3β-HSD activity. Leydig cell preparations from immature and from mature rats contained 43 ± 7 (4) and 80 ± 7 (4) percent 3β-HSD positive cells, respectively. The number of attached cells was calculated as described in appendix paper I in case of the mature preparation, whereas in preparations from immature rats the number of added cells was used since more than 90% of the cells were attached. Pregnenolone concentrations were measured in the media by radioimmunoassay as described by Rommerts et al. (1982).
Means ± SD of 5 different cell preparations are given.
*significantly different from 21-23 days ($p<0.005$).

production. Moreover, the pregnenolone productions in the presence of 22-R-OH-cholesterol show that not only the basal and LH-regulated steroid production, but also that the capacity of the CSCC-enzyme complex has increased during maturation.

V.3.2. Pregnenolone conversion

During maturation a shift in pregnenolone metabolism occurs, resulting in a higher testosterone production in mature animals. We have used the conversion of pregnenolone to testosterone (expressed as the ratio (testosterone production : pregnenolone production) x 100% = $\frac{T}{P}$ (%)) as a parameter which indicates the "maturity" of the Leydig cell. As is shown in Fig. V.4. the percentage of pregnenolone which is converted to testosterone by Leydig cells in vitro increases with increasing age of the animals. The percentage pregnenolone converted to testosterone by Leydig cell preparations from 35-40-day-old rats is in between the percentage conversion by cells from immature and mature rats. Whether this is caused by a mixture of "immature" and "mature" cells, or by cells in an "intermediate" state, cannot be answered yet.

V.3.3. Non-specific esterase activity

Leydig cells from mature rats can be distinguished from Leydig cells from immature rats by the difference in non-specific esterase activity (see appendix paper II). We have characterized Leydig cell preparations from rats of different ages by histochemical staining for 3ß-HSD activity (as a marker for both "immature" and "mature" Leydig cells) and for α-Naphtyl Esterase (α-NE) (as a marker for "mature" Leydig cells).

The results given in Table V.II. show that α-NE positive cells, which are absent in preparations from immature rats, appear during maturation. Cells which showed α-NE activity always showed 3ß-HSD activity as well. This has led to an

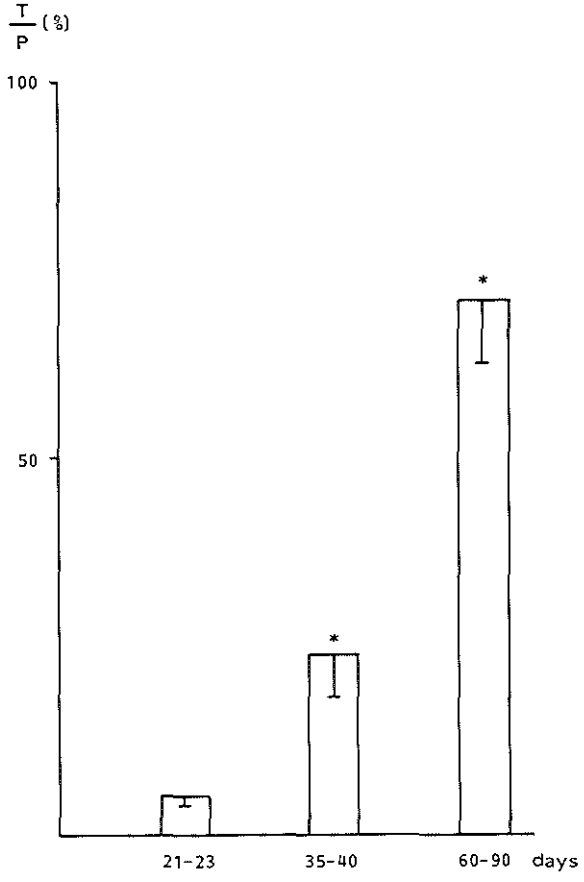


Fig. V.4. Developmental changes in pregnenolone metabolism. Leydig cell preparations were obtained from rats of different ages by Ficoll purification as described in appendix paper I. After 60 min incubation without additions, cells were incubated either in the presence of inhibitors of pregnenolone metabolism for pregnenolone measurements or in parallel incubations without inhibitors for testosterone measurements. Pregnenolone and testosterone concentrations were measured in the media by radioimmunoassay as described by Rommerts et al. (1982). The ratio (produced testosterone : produced pregnenolone) x 100% = T/P(%)) was calculated.
Means ± SD of 4 different cell preparations.
*significantly different from preceding age group ($p<0.005$).

almost 100% overlap between 3β-HSD positive and α-NE positive cells in preparations from 60-90-day-old rats. In preparations from 35-40-day-old rats, 2 types of Leydig cells are present, viz. 3β-HSD positive cells which are α-NE negative, representing "immature" Leydig cells, and 3β-HSD positive cells which are α-NE positive as well, representing "mature" Leydig cells.

Table V.II. Occurrence of 3β-HSD positive and α-NE positive cells in Leydig cell preparations from rats of different ages.

age in days	percentage 3β-HSD positive cells	percentage α-NE positive cells
21-23	43 ± 7	0 ± 0
35-40	73 ± 11	56 ± 13
60-90	80 ± 7	79 ± 9

Leydig cell preparations were obtained from rats of different ages by Ficoll purification as described in appendix paper I. After 60 min incubation 3β-HSD and α-NE (30 sec) histochemistry was performed as described in appendix papers I and II, and the percentage positive cells was estimated.
Means ± SD of 5 different cell preparations.

V.4. CONCLUSIONS

Our data show that during maturation the percentage Leydig cells in the interstitium increases, probably as the result of differentiation from mesenchymal precursor cells. Macrophages which were almost absent in the interstitium from immature rats, formed approximately 20% of the interstitial cells in mature rats. Furthermore, there are indications that during differentiation the Leydig cells acquire their morphological characteristics previous to their steroidogenic activities. This will contribute to a heterogeneous Leydig cell population

at this stage of development.

The steroidogenic capacity of Leydig cells and the proportion of pregnenolone which is converted to testosterone increases during maturation. Esterase activity (a marker for "mature" Leydig cells) was present in a fraction ot the cells isolated from 30-40-day-old rats, whereas other 3β-HSD positive cells were still esterase negative, thus reflecting a heterogeneous population of Leydig cells at least with respect to the presence of this enzyme.

Hence, the interstitial cell preparations from maturing rats are probably heterogeneous due to the presence of Leydig cells in different stages of development.

CHAPTER VI
GENERAL DISCUSSION

Properties of Leydig cells can vary between different species and also within one species depending on the reproductive activity of the testis (see Chapter I.3.2.). Moreover, there is evidence that different Leydig cells present in the same testis also show different properties (see Chapter I.5.2.). The studies described in this thesis partly deal with this so-called "heterogeneity" and the results will be discussed in Chapter VI.1. In addition, studies have been performed concerning the origin and differentiation of Leydig cells and the role of the macrophages in the testis (see Chapter VI.2.). Finally, a possible involvement of Leydig cells in the immunosuppressive activity of the testis will be discussed in Chapter VI.3.

VI.1. HETEROGENEITY OF LEYDIG CELLS

Several investigators (Payne et al., 1980; Chen et al., 1981; Cooke et al., 1981; O'Shaugnessey et al., 1981) have demonstrated for mature rats that Leydig cells showing the same number of LH receptors can be separated into subpopulations which differ in steroidogenic properties. These observations suggest that the population of Leydig cells in mature rats is functionally heterogeneous. However, our results show (see Chapter II.2.) that Leydig cells can be damaged severely during the isolation procedure. This damage varied from damage to the membrane, as was demonstrated by a permeability to NAD(P)H, to total cell disruption resulting in the release of microsomal enzymes. It appeared that the final cell preparation contained intact cells, which showed a good stimulation of testosterone production by LH, in addition to damaged cells, which showed no response to LH. Consequently, the damage occurring during isolation of the cells can explain that the isolated population of Leydig cells was heterogeneous with respect to steroid

production. Moreover, the final yield of cells was very low (approximately 10%) and it is doubtful whether this fraction may be considered as representative for the Leydig cell population present *in vivo*. Since the authors mentioned above use essentially the same isolation procedure, it seems likely that the heterogeneity which they observed is at least partly an artifact.

Some pilot experiments were performed using a Fluorescence Activated Cell Sorter in order to separate Leydig cells into subpopulations based on differences in the amount of LH or LHRH receptors, or in cell size. These experiments appeared unsuccessful, probably partly because of the presence of damaged cells and partly because of practical problems with the technique of cell labelling. Knowing that the Leydig cell preparation contained damaged cells and was only a fraction of the total cell population, we have not continued these attempts to obtain different subpopulations of Leydig cells. Hence, we could not investigate the functional properties of different subpopulations of Leydig cells and we can draw no conclusions about a possible heterogeneity of Leydig cells in testes from mature rats *in vivo*. Yet, several other observations described in this thesis in combination with data from other authors, support the view that the population of Leydig cells *in vivo* may be heterogeneous.

We have demonstrated that properties of Leydig cells change during maturation. The steroidogenic capacity expressed per Leydig cell increases as well as the percentage of pregnenolone which is converted to testosterone. This process of maturation may occur in all Leydig cells at the same time which, in a maturing rat, results in a population of cells, all being in an "intermediate" stage. As another possibility we have considered that at a specific time a fraction of the Leydig cells has already the characteristics of "mature" Leydig cells, whereas other cells are still in the "immature" stage, resulting in a heterogeneous population of Leydig cells. Observations on other characteristic properties of "mature"

Leydig cells, support the latter possibility. For example, Clausen et al. (1981) have demonstrated that the number of LH-receptors per cell increases during maturation, in such a way that a population with a low number of receptors changes into a population with a high number of receptors, resulting in the presence of two separate populations of Leydig cells differing in receptor number in the period in between. We have made similar observations on the presence of esterase activity (see Chapter V.3.3.). Esterase activity develops in the Leydig cells during maturation, but not all cells acquire esterase activity at the same time. This causes the presence of two types of Leydig cells in the maturing rat, those which are already esterase positive and those which are still esterase negative. All these observations support the view that in the maturing rat the Leydig cell population is heterogeneous with respect to certain properties.

In Leydig cell preparations from immature rats we have observed that the number of Leydig cells characterized by morphological criteria is higher than the number characterized by 3ß-Hydroxy Steroid Dehydrogenase activity. This discrepancy may indicate that Leydig cells acquire the morphological characteristics of a Leydig cell before the steroidogenic activities have developed. Taken together, these data indicate that during development the Leydig cell population is heterogeneous as the result of differences in stages of differentiation and maturation.

Since there is very little known about the turnover of Leydig cells after sexual maturation, it is not clear to which extent these differences in differentiation and maturation contribute to the heterogeneity of the Leydig cell population present in testes from mature rats. In mature rats we have found, however, that LH-dependent precursor cells for Leydig cells are present, which can develop into a new population of Leydig cells when the existing Leydig cells are destroyed by treatment with Ethylene Dimethane Sulfonate (EDS) (see Chapter IV.2.). This implies that testes from mature rats still have

the capacity to develop Leydig cells and it may be possible, therefore, that differentiation of Leydig cells also takes place in the normal mature rat testis, albeit at a low rate.

There are indications that properties of existing differentiated Leydig cells can be modulated by interaction with other cell types, and this may also be the cause of a heterogeneous population of Leydig cells. For example, Bergh (1982) has demonstrated that the size of Leydig cells can vary depending on the stage of the spermatogenic cycle in the adjacent tubules, viz. Leydig cells near tubules in stages VII - VIII are larger than Leydig cells near tubules in stages IX - XIV. Another cell type which may modulate Leydig cell function is the macrophage. The macrophages present in the interstitium can be in very close association with the Leydig cells (Connell & Christensen, 1975; Miller et al., 1983; Bergh, 1985). Since the presence of various steroidogenic enzymes was demonstrated in macrophages (Milewich et al., 1982, 1983; Lofthus et al., 1984) and it was suggested by Vernon-Roberts (1969) that the macrophages in the adrenal contribute largely to the 17α-hydroxylation of the steroids, we have investigated the possibility that testicular macrophages are involved in steroid production by the testis. We have found that macrophages *in vitro* are not active in pregnenolone production and show no significant 17α-hydroxylase activity. These data suggest that macrophages per se are not active in steroidogenesis. However, Yee & Hutson (1985) have shown that conditioned medium obtained from testicular macrophages stimulated the steroid production by Leydig cells *in vitro* and that this stimulating effect was doubled when macrophages were preincubated with FSH. This suggests that macrophages may also modulate Leydig cell function *in vivo* under the influence of FSH. It seems likely, therefore, that Leydig cells which are associated with macrophages may differ in their steroidogenic activities from Leydig cells which are not directly in contact with macrophages. Hence, macrophages may contribute to the heterogeneity within the population of Leydig cells in mature

rats.

VI.2. ORIGIN AND DEVELOPMENT OF LEYDIG CELLS

The development of Leydig cells has been the subject of many studies. These studies have been performed in normal maturing rats where the testis as a whole undergoes many developmental changes. We have studied the development of Leydig cells after sexual maturation using mature rats which were experimentally depleted of Leydig cells via administration of Ethylene Dimethane Sulfonate (EDS) (see Chapter IV). We observed that the reappearance of Leydig cells in these mature rats was dependent on LH and not on FSH. This is in clear contrast to the development of Leydig cells during normal sexual maturation where FSH is required for a normal differentiation of the Leydig cells (Odell et al., 1973; van Beurden et al., 1976). Kerr & Sharpe (1985[a,b]) even suggested that FSH, and not LH, is the major factor responsible for the differentiation of Leydig cells. Since the Sertoli cell is the main target for FSH, this difference in regulation of Leydig cell differentiation between immature and mature rats may indicate either that the development of Leydig cells in mature rats is independent of products secreted by the Sertoli cells or that Sertoli cells in mature rats secrete these products autonomously and independent of FSH.

We have observed also that the new population of Leydig cells in EDS-treated mature rats originates from precursor cells which do not show the morphological characteristics of Leydig cells. Since Clegg & McMillan (1965) have suggested that macrophages may transform into "Leydig-like" cells and because the macrophages remain in the testis after treatment with EDS, we have investigated the possibility that these cells give rise to the new population of Leydig cells. However, when the macrophages in the testis were labelled with trypan blue or fluorescent beads previous to the EDS treatment, this label was not present in the newly formed Leydig cells. Hence, we have

rejected the hypothesis that macrophages are the precursor cells for Leydig cells.

Another, more likely candidate to be the precursor cell is the mesenchymal cell. Support for this hypothesis is obtained from the following observations. Our data demonstrate that the repopulation of Leydig cells after EDS treatment is dependent on LH and, in hypophysectomized rats, can be stimulated with hCG. Kerr et al. (1985[b]) have shown that interstitial cell preparations obtained from adult mice contain a fraction of mesenchymal cells which specifically bind hCG to their surface. Furthermore, in the testicular interstitium from immature rats a substantial number of mesenchymal cells is present in addition to Leydig cells (Laws, 1985; Kerr & Sharpe, 1985[b]) (see Chapter V.2.). Kerr & Sharpe (1985[b]) have demonstrated that stimulation of the formation and differentiation of Leydig cells is correlated with a decrease in the number of these mesenchymal cells.

It seems most likely, therefore, that during normal sexual maturation mesenchymal cells differentiate into Leydig cells and that a similar process may occur in mature rats after experimental depletion of Leydig cells.

VI.3. ARE LEYDIG CELLS INVOLVED IN IMMUNOSUPPRESSION?

In addition to the Leydig cells, which form the majority of the cells present in the testicular interstitium from mature rats, a significant number of macrophages is present. As is described in Chapter III, there were indications from the literature that Leydig cells and macrophages may show some functional similarities. The possible role of the macrophages in the steroid production of the testis has already been discussed in Chapter VI.1. In this section the possible involvement of the Leydig cells in immunological processes will be discussed.

It has been demonstrated (Born & Wekerle, 1981; Rivenson et al., 1981) that lymphocytes can bind specifically to the

Leydig cell membrane surface. We have shown that the Leydig cells from mature rats contain a membrane antigen which is considered specific for macrophages as well as F_c-receptors, a characteristic of macrophages. It seems possible that these membrane properties play a role in the specific binding of lymphocytes. At this moment the meaning of this observation is still not clear, but it may relate to the immunosuppressive activity of the testis, as observed by Head et al. (1983). They have found that parathyroid allografts could survive beneath the capsule of the testis, but were rejected after transplantation beneath the capsule of the kidney. Born & Wekerle (1982) have shown that Leydig cells in co-culture with lymphocytes suppress lymphocyte proliferation, which suggests that the Leydig cells are involved in the immunosuppression of the testis. This is supported by observations that steroids (Staples et al., 1983) and prostaglandins of the E-class (Stenson & Parker, 1980) inhibit lymphocyte proliferation. Moreover, Strom & Carpenter (1983) have demonstrated that prostaglandin E_1 counteracts the rejection of allografts. Taken together, these data suggest that Leydig cells, which are the main source of the steroids in the testis and which are clearly involved in the production of prostaglandins (see Chapter III.2.5.), may play an important role in the immunosuppressive activity of the testis.

VI.4. CONCLUSIONS

1. Heterogeneity of Leydig cells in preparations isolated from mature rats reflects in part an artifact, resulting from the isolation procedure.
2. The population of Leydig cells present in maturing rats is probably heterogeneous as the result of differences in the stage of differentiation and maturation, but it is unknown whether and to which extent this also leads to a heterogeneity of Leydig cells in mature rats.
3. Macrophages, which are not active in steroid production

themselves, seemed to be involved in regulation of steroid production by Leydig cells and may, via local interactions with Leydig cells, contribute to the heterogeneity of Leydig cells in mature rats.

4. We have presented evidence that Leydig cells could originate from mesenchymal precursor cells. This precursor cell is still present after maturation and can give rise to a new population of Leydig cells under pathological conditions when the existing population of Leydig cells has been destroyed.
5. In mature rats the Leydig cells are involved in the production of large amounts of prostaglandin E_2. This product, possibly in combination with the steroids produced by the Leydig cells, may play a role in the immunosuppressive activity of the testis.

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SUMMARY

The testes in mammals consist of two main compartments, viz. the seminiferous tubules, in which spermatogenesis takes place and the interstitium. Various cell types are present in the interstitium: Leydig cells, macrophages, fibroblasts, myoid cells, endothelial cells and mesenchymal cells. The Leydig cells are the source of the androgen production.

There are indications that the population of Leydig cells present in the rat testis is heterogeneous. Differences in cell size, number of LH receptors and 3β-Hydroxy Steroid Dehydrogenase (3β-HSD) activity have been reported. Furthermore, subpopulations of Leydig cells, showing the same number of LH receptors, with different steroidogenic activities have been isolated. Our results described in Chapter II.2. and appendix paper I show that a part of the Leydig cells isolated from testes of mature rats was disrupted during the isolation procedure and that the final cell preparation contained damaged cells which do not respond to LH, as well as intact cells which can be stimulated by LH. Hence, Leydig cell preparations isolated from testes of mature rats are heterogeneous with respect to steroid production, which is at least partly the result of an artifact. Pilot experiments were performed, using a Fluorescence Activated Cell Sorter, to separate subpopulations of Leydig cells based on differences in their cell size, the number of LH or LHRH receptors and the content of smooth endoplasmic reticulum (see Chapter II.3.). These attempts were not very successful, probably due to 1) the poor quality of the cell preparation, 2) problems with the technique of labelling and 3) an effect of the dye on steroidogenic activities.

The interstitium of the testes from mature rats contains in addition to Leydig cells approximately 15-20% macrophages. It has been shown that Leydig cells and macrophages are often very closely associated, but very little was known about the function of the testicular macrophages. The presence of several

steroid converting enzymes in peritoneal, alveolar and adrenal macrophages, was interpreted as an indication that macrophages could be involved in steroid production. The experiments described in Chapter III.2.1. and III.2.2. have shown that testicular macrophages are not active in pregnenolone production in vitro and show a 17α-hydroxylase activity which is insignificant when compared to Leydig cells. Leydig cells from mature rats showed a much higher non-specific esterase activity (an enzyme which is generally used as a marker for macrophages) than testicular macrophages (see Chapter III.2.3. and appendix paper II). We have concluded, therefore, that non-specific esterase in the testis is a more specific marker for Leydig cells than for macrophages. In addition we have found some similarities between Leydig cells and macrophages. Leydig cells contain a membrane antigen which is considered as specific for macrophages and it is very likely that they also contain F_c-receptors (see Chapter III.2.4.). Prostaglandin E_2 (PGE_2) (one of the secretion products of macrophages) was demonstrated in testicular tissue from both normal and Leydig Cell Depleted (LCD) mature rats. An extensive increase in the PGE_2 content after administration of hCG, however, was found only in the testes from normal rats and not from LCD rats (see Chapter III.2.5.). This demonstrates that the Leydig cells are involved in PGE_2 production by the testis.

In Chapter IV we have described our results on the regeneration of Leydig cells in mature rats after selective destruction of the existing Leydig cells. Administration of Ethylene Dimethane Sulfonate (EDS) to mature rats resulted in a specific destruction of Leydig cells (see Chapter IV.2. and appendix paper III). Repopulation of Leydig cells occurred and appeared to be dependent on LH and not on FSH (see Chapter IV.3. and appendix paper IV). This is in contrast to the differentiation of Leydig cells during normal sexual maturation which is regulated by both LH and FSH.

The composition and the properties of the interstitial cells of the testis change during maturation (see Chapter V).

The results in Chapter V.1. show that the interstitial tissue from immature rats, and also the isolated interstitial cell preparation, contain besides Leydig cells a significant number of mesenchymal cells and hardly any macrophages. In the interstitium from mature rats the majority of the cells are Leydig cells, but rather large numbers of macrophages are also present. The isolated cell preparation contained approximately 80% Leydig cells and 20% macrophages. The experiments described in Chapter V.2. showed that the mean steroidogenic capacity of Leydig cells isolated from mature rats was significantly higher than that of Leydig cells from immature rats. The percentage pregnenolone which was converted to testosterone gradually increased during maturation. Concomitantly, non-specific esterase activity developed in the Leydig cells.

In the general discussion (Chapter VI) the results described in this thesis have been discussed with respect to: heterogeneity of Leydig cells (Chapter VI.1.); origin of Leydig cells (Chapter VI.2.) and the possible role of the Leydig cells in the immunosuppressive activity of the testis (Chapter VI.3.). Briefly, we have concluded that:

1) the heterogeneity with respect to steroid production in Leydig cell preparations obtained from mature rats is at least partly the result of an artifact.
2) the Leydig cell population from maturing rats is heterogeneous probably due to differences in differentiation and maturation of the individual Leydig cells, but it is not clear whether this also contributes to the heterogeneity of Leydig cell populations from mature rats.
3) the testicular macrophages, which as such are not active in steroid production, may stimulate the steroid production by Leydig cells. Hence, Leydig cells which are in contact with macrophages may differ in properties from Leydig cells which are not in contact with macrophages.
4) Leydig cells probably originate from a precursor cell of mesenchymal origin. This seems true for both normal development of Leydig cells and for development of Leydig

cells in testes of mature rats after depletion of Leydig cells.

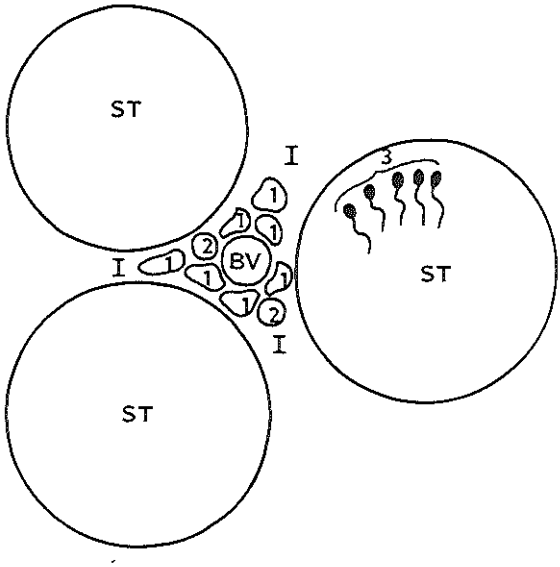
5) the Leydig cells may play a role in the immunosuppressive activity of the testis, since these cells are the main source of steroids and are involved in the production of prostaglandin E_2.

SAMENVATTING

Deze samenvatting is er op gericht het onderzoek dat beschreven is in dit proefschrift ook voor diegenen die niet bekend zijn met het "testis-onderzoek" begrijpelijk te maken. Dit brengt met zich mee dat bepaalde feiten weleens op een iets eenvoudiger manier zijn weergegeven dan strikt genomen juist is.

Het mannelijke voortplantingsorgaan, de testis (ook wel testikel of zaadbal genoemd), bestaat bij zoogdieren uit 2 afzonderlijke compartimenten, nl. de tubuli seminiferi (zaadvormende buisjes), waarin de spermatozoa (zaadcellen) gevormd worden en het interstitium (tussenliggende weefsel) waarin het "mannelijk" hormoon, testosteron, wordt gevormd. Testosteron is noodzakelijk voor de rijping van de zaadcellen en verantwoordelijk voor de mannelijke geslachtskenmerken. De opbouw van de testis is schematisch weergegeven in de figuur op de volgende pagina. In het interstitium bevinden zich voornamelijk Leydig cellen (genoemd naar de ontdekker Franz Leydig), die het testosteron produceren. Daarnaast worden ook macrofagen, cellen die normaal vooral een "opruim funktie" hebben in het lichaam, vrij frequent aangetroffen. Verder worden er kleinere en grotere bloedvaten aangetroffen in het interstitium.

De testosteron produktie door de Leydig cellen staat onder invloed van het hormoon LH (lutropine) dat in de hypofyse (hersenaanhangsel) gemaakt wordt en via het bloed naar de testis wordt getransporteerd. Leydig cellen kunnen o.a. herkend worden op grond van hun uiterlijke kenmerken (morfologie) of door de aanwezigheid van bepaalde zgn. marker-enzymen, welke zichtbaar gemaakt kunnen worden d.m.v. een chemische reaktie. Het 3β-Hydroxy Steroid Dehydrogenase (3β-HSD), een enzym dat essentieel is voor de vorming van testosteron, is zo'n marker-enzym waarmee Leydig cellen kunnen worden aangetoond.



Schematische doorsnede door de testis.
ST=Seminifere tubuli; I=Interstitium; BV=bloedvat
1. Leydig cellen; 2. macrofagen; 3. zaadcellen

Er zijn aanwijzingen dat niet alle Leydig cellen hetzelfde zijn. Ze kunnen bijvoorbeeld verschillen vertonen met betrekking tot de hoeveelheid testosteron die geproduceerd wordt, de gevoeligheid voor het hormoon LH en de aktiviteit van het enzym 3ß-HSD. Een dergelijk verschijnsel, dat cellen behorend tot eenzelfde celtype verschillen in eigenschappen vertonen, noemt men heterogeniteit. Heterogeniteit binnen een celpopulatie kan o.a. het gevolg zijn van een verschil in differentiatie (mate van ontwikkeling) of lokalisatie (ligging t.o.v. andere cellen), waardoor de cellen beinvloed kunnen worden, maar in lang niet alle gevallen is de oorzaak bekend. In het werk beschreven in dit proefschrift is o.a. het voorkomen van heterogeniteit binnen de populatie van Leydig cellen aanwezig in de testis van de rat onderzocht en is geprobeerd subpopulaties van verschillende Leydig cellen in handen te krijgen.

In het interstitium van de testis bevinden zich naast

Leydig cellen ook macrofagen. De aanwezigheid van deze macrofagen, die soms in zeer nauw kontakt staan met Leydig cellen, is reeds door verschillende auteurs beschreven, maar er is nog erg weinig bekend over de funktie van deze cellen in de testis. Aangetoond is dat bepaalde eigenschappen die normaal aan macrofagen worden toegeschreven (zgn. markers) ook aanwezig zijn in Leydig cellen en ook het omgekeerde is het geval. In dit onderzoek is met behulp van een aantal specifieke markers voor Leydig cellen en voor macrofagen nagegaan of, en in hoeverre, Leydig cellen en testiculaire macrofagen dezelfde funktionele eigenschappen vertonen.

Tevens is aandacht besteed aan de oorsprong en ontwikkeling van Leydig cellen. Het is bekend dat het aantal Leydig cellen sterk toeneemt gedurende de puberteit. Het is echter niet bekend welke cellen als "voorlopers" van de Leydig cellen moeten worden beschouwd. Ook is het nog onduidelijk of, en met welke snelheid, in het volwassen individu Leydig cellen verdwijnen en of nieuwe cellen worden gevormd. Door gebruik te maken van een stof die selektief de Leydig cellen in de testis van volwassen ratten vernietigt, was het mogelijk na te gaan of er een nieuwe aanmaak van Leydig cellen plaatsvond en welke faktoren hierbij een rol speelden.

Experimenten over het voorkomen van heterogeniteit binnen de populatie Leydig cellen verkregen uit testes van volwassen ratten zijn beschreven in hoofdstuk II. Het is gebleken dat tijdens de isolatieprocedure, waarbij de Leydig cellen uit het testisweefsel worden vrijgemaakt, een groot deel van de cellen wordt beschadigd. Een deel van deze cellen bleek totaal vernietigd, waarbij de gehele celinhoud vrijkwam, en een deel van de cellen verkeerde in een "aangeslagen" toestand. Deze "aangeslagen" cellen waren niet meer in staat te reageren op het hormoon LH dat normaal de produktie van testosteron door de Leydig cel stimuleert. Daarnaast bevatte het preparaat ook een fraktie intakte (onbeschadigde) cellen die wel reageerden op stimulatie met LH. Dit betekent dat het geisoleerde

celpreparaat 2 typen Leydig cellen bevat, nl. een type dat wel gevoelig is voor LH (de intakte celfraktie) en een type dat ongevoelig is voor LH (de beschadigde celfraktie). Het preparaat is dus heterogeen. Deze heterogeniteit is het gevolg van de behandeling van de cellen en moet dus beschouwd worden als een artefakt. Er is geprobeerd om uit dit preparaat verschillende typen van Leydig cellen van elkaar te scheiden en zodoende subpopulaties te verkrijgen die wellicht funktioneel verschillend waren. Hiervoor is gebruik gemaakt van fluorescerende stoffen waarmee de cellen gelabeld werden. Deze pogingen waren echter niet erg succesvol. De volgende faktoren hebben hierbij waarschijnlijk een belangrijke rol gespeeld: 1) de slechte kwaliteit van het celpreparaat, 2) problemen met de techniek van labelling en 3) beinvloeding van de aktiviteit van de Leydig cellen door de labelling.

In hoofdstuk III is nagegaan in hoeverre Leydig cellen en macrofagen verkregen uit testes van volwassen ratten een overeenkomst in eigenschappen vertonen. Gebleken is dat macrofagen niet aktief zijn in het produceren van pregnenolon, een verbinding waaruit het testosteron wordt gevormd, en dat de aktiviteit van de enzymen 3ß-HSD en 17α-hydroxylase, beide noodzakelijk voor de vorming van testosteron uit pregnenolon, verwaarloosbaar is. De aktiviteit van deze enzymen was daarentegen in Leydig cellen erg hoog. Hieruit is gekonkludeerd dat de Leydig cellen de belangrijkste bron zijn van testosteron en dat de macrofagen hieraan waarschijnlijk geen belangrijke bijdrage leveren. Esterase, een enzym dat vaak gebruikt wordt voor het karakteriseren van macrofagen bleek een veel hogere aktiviteit te vertonen in Leydig cellen. Dit impliceert dat esterase in de testis een betere marker is voor Leydig cellen dan voor macrofagen. Het blijkt dus dat Leydig cellen en macrofagen duidelijk in eigenschappen verschillen. Toch zijn er ook resultaten die een overeenkomst in eigenschappen suggereren. Macrofagen kunnen gekarakteriseerd worden door de aanwezigheid van bepaalde membraan-antigenen (strukturen aanwezig op het celoppervlak), waaronder bv. de zgn.

F_c-receptor. Onze resultaten vormen een aanwijzing dat ook op Leydig cellen enkele van deze antigenen aanwezig zijn. De funktie hiervan is echter nog onduidelijk. Daarnaast is gebleken dat de stof prostaglandine waarvan bekend is dat deze o.a. door macrofagen wordt geproduceerd in grote hoeveelheden in de testis aanwezig is en dat de produktie afhankelijk is van de aanwezigheid van Leydig cellen. Dit suggereert dat Leydig cellen aktief zijn in de produktie van prostaglandines. Het is echter ook mogelijk dat de Leydig cellen zelf geen prostaglandines produceren, maar via een signaal de macrofagen tot produktie aanzetten.

In hoofdstuk IV zijn de resultaten beschreven over de vorming van Leydig cellen in de testes van volwassen ratten. Wij hebben aangetoond dat behandeling van volwassen ratten met de stof Ethyleen Dimethaan Sulfonaat (EDS) leidt tot een selektieve vernietiging van alle Leydig cellen in de testis. Daarna treedt er een herstel op en worden er nieuwe Leydig cellen gevormd. Dit proces blijkt afhankelijk te zijn van het hormoon LH. Hieruit zijn de volgende 2 konklusies getrokken: 1) een volwassen testis heeft nog de kapaciteit om een nieuwe populatie van Leydig cellen te vormen en 2) de voorloper cel van deze nieuwe populatie, welke niet als Leydig cel herkenbaar is, is bestand tegen het EDS en gevoelig voor LH.

Eigenschappen van Leydig cellen en de samenstelling van het interstitium veranderen gedurende de puberteit. In het eerste deel van hoofdstuk V zijn de samenstelling van het interstitiële celpreparaat van onvolwassen en volwassen ratten met elkaar vergeleken. De cellen geisoleerd uit testes van volwassen ratten bestaan vrijwel uitsluitend uit Leydig cellen (3ß-HSD positieve vellen) (ca. 80%) en macrofagen (ca. 20%). Het preparaat van onvolwassen ratten, daarentegen, bevat slechts ca. 45% Leydig cellen (3ß-HSD positieve cellen) en nauwelijks macrofagen. De resterende fraktie bevat een zeer gering percentage endotheelcellen (cellen die de wand van een bloedvat vormen) en verder cellen die in het algemeen worden beschouwd als "mesenchymale" cellen (d.w.z. cellen

afkomstig van het embryonale bindweefsel). Het is zeer waarschijnlijk dat deze fraktie de voorloper cellen van de Leydig cellen bevat. De resultaten beschreven in het tweede deel van hoofdstuk V tonen aan dat Leydig cellen van volwassen ratten veel meer pregnenolon (een voorloper van het testosteron) kunnen produceren dan Leydig cellen van onvolwassen ratten. Bovendien neemt het percentage pregnenolon dat wordt omgezet in testosteron toe gedurende de ontwikkeling. Dit gaat gepaard met het verschijnen van esterase aktiviteit, hetgeen als een marker voor volwassen Leydig cellen kan worden beschouwd.

In hoofdstuk VI zijn de in dit proefschrift beschreven resultaten bediskussieerd in het bijzonder met betrekking tot: heterogeniteit van Leydig cellen, de oorsprong van Leydig cellen en een eventuele rol van de Leydig cellen bij de immuun-suppressie (het onderdrukken van afstotingsreakties) in de testis. Dit heeft geleid tot de volgende konklusies:

1) De heterogeniteit die wordt waargenomen in een Leydig cel preparaat verkregen uit testes van volwassen ratten is in elk geval ten dele het gevolg van de behandeling en dus een artefakt. Het voorkomen van een reële heterogeniteit wordt niet uitgesloten. Het lijkt waarschijnlijk dat de populatie Leydig cellen in een zich ontwikkelende rat heterogeen is als gevolg van de aanwezigheid van Leydig cellen in verschillende stadia van volwassenheid. In hoeverre dit ook een rol speelt bij reeds volwassen ratten is nog onduidelijk. Onze resultaten hebben aangetoond dat macrofagen zelf geen testosteron produceren. Er zijn echter aanwijzingen in de literatuur dat macrofagen wel in staat zijn de testosteron produktie van Leydig cellen te stimuleren. Niet alle Leydig cellen staan in kontakt met macrofagen en het is daarom mogelijk dat de funktie van bepaalde Leydig cellen wordt beinvloed door macrofagen en van andere Leydig cellen niet. Dit zou kunnen leiden tot een funktionele heterogeniteit.

2) Cellen van "mesenchymale" oorsprong zijn waarschijnlijk de voorlopers van de latere Leydig cellen. Deze voorloper cellen zijn ook in de testes van volwassen ratten nog aanwezig en zijn in staat een nieuwe populatie van Leydig te vormen wanneer de bestaande populatie cellen kunstmatig is vernietigd.
3) Het lijkt mogelijk dat de Leydig cellen, als belangrijkste bron van steroiden in de testis en betrokken bij de produktie van prostaglandines, een rol spelen bij de onderdrukking van de afstotingsreaktie in de testis. Het is namelijk aangetoond dat wanneer weefsel van een ander individu getransplanteerd wordt in de testis, de afstotingsreaktie daar geringer is dan elders in het lichaam. Er zijn aanwijzingen dat steroiden (een groep van verbindingen waar ook het testosteron toe behoort) en ook prostaglandines, een afweerreaktie kunnen onderdrukken.

LIST OF ABBREVIATIONS

CSCC	Cholesterol Side Chain Cleavage
dbc-AMP	dibutyryl cyclic AMP; N^6-2'-O-dibutyryl-adenosine-3':5'-monophosphate
EDS	Ethylene Dimethane Sulfonate
FSH	Follicle Stimulating Hormone (follitropin)
hCG	human Chorionic Gonadotropin
3β-HSD	3β-Hydroxy Steroid Dehydrogenase
LCD	Leydig Cell Depleted
LH	Luteinizing Hormone (lutropin)
LHRH	Luteinizing Hormone Releasing Hormone (luliberin)
NADP(H)	(reduced) Nicotinamide Adenine Dinucleotide Phosphate
α-NE	α-Naphtyl Esterase
PGE_2	prostaglandin E_2

SLOTWOORD

Bij het voltooien van dit proefschrift wil ik graag iedereen bedanken die hiertoe heeft bijgedragen.

In de eerste plaats geldt dit mijn promotor, Henk van der Molen, die ik wil bedanken voor zijn begeleiding en suggesties t.a.v. het onderzoek. Bij het kritisch doorlezen van de manuscripten en het proefschrift heb ik vooral ook veel profijt gehad van zijn gedegen kennis van het Engels.

Mijn werkleider, Focko Rommerts, ben ik dankbaar voor de wijze waarop hij het onderzoek heeft begeleid. Zijn praktische suggesties en nooit aflatende stroom van ideeën alsmede de prettige samenwerking en de vele stimulerende diskussies zijn voor mij een grote steun geweest. We zijn het eens over het feit dat de menselijke populatie "heterogeen" is; is het dan vreemd dat we het ook wel eens niet eens waren?

Prof. Jongkind, mijn tweede promotor, ben ik zeer erkentelijk voor zijn "celbiologische" inbreng in het onderzoek en voor het zorgvuldig doorlezen en korrigeren van het proefschrift.

Prof. Van der Werff ten Bosch en Prof. Wensing wil ik bedanken voor het snel en kritisch doorlezen van het proefschrift.

De (oud) KLM-ers Jos Hoogerbrugge, Paul Reuvers, Ria van der Heyden en Hilda Kornman ben ik dankbaar voor hun hulp bij de isolatie van Leydig cellen, radioimmunoassays en esterase-bepalingen.

Verder wil ik ook een aantal mensen buiten de afdeling Biochemie II bedanken voor hun bijdrage aan dit proefschrift. Van de afdeling Celbiologie: Ton Verkerk voor zijn assistentie bij de bediening van de "cell sorter" en Pim Visser voor het maken van de EM-coupes van testispreparaten. Dr. Vincent en Freek Zijlstra, van de afdeling Farmakologie, voor hun bijdrage aan het prostaglandine onderzoek in de testis. Jan Rozing en Frans Tielen, van de afdeling Inwendige Geneeskunde I, die mij in de gelegenheid stelden vele macrofaag-antilichamen op Leydig

cellen te testen. Dick de Rooij van de afdeling Celbiologie van de medische faculteit te Utrecht voor zijn samenwerking in het EDS-onderzoek.

Graag wil ik ook hen bedanken die het uiterlijk van dit proefschrift hebben verzorgd. Marja Decae en Rosemarie van der Ploeg die met grote snelheid en zorgvuldigheid de gehele tekst typten. Tar van Os voor het afdrukken van de foto's in het proefschrift en op de voorzijde, waarbij hij het maximum wist te halen uit de negatieven die soms van minimale kwaliteit bleken te zijn. Paul Reuvers voor het ontwerpen en samenstellen van de achterzijde van de omslag, die een kleine selektie laat zien uit de vele foto's die gemaakt zijn voordat het beoogde doel werd bereikt.

Met name wil ik ook hier mijn beide paranimfen, Els Berns en Axel Themmen noemen die ik dankbaar ben voor de steun die zij verleend hebben, niet alleen bij de organisatie van de feestelijke kant van de zaak, maar ook op vele momenten in de onderzoek- en schrijffase daaraan voorafgaand.

Tevens wil ik hier mijn ouders bedanken die mij in de gelegenheid gesteld en gestimuleerd hebben te gaan studeren en altijd erg hebben meegeleefd.

Tot slot wil ik graag alle collega's en vrienden bedanken die niet met name genoemd zijn en die ook een bijdrage hebben geleverd aan het tot stand komen van dit proefschrift.

Rinkje

CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 7 september 1954 te Rotterdam geboren. Na de opleiding gymnasium ß aan de Libanon Scholengemeenschap te Rotterdam volgde in 1973 de studie biologie aan de Rijks Universiteit te Utrecht. Na het kandidaatsexamen medische-biologie (B5*) in januari 1977, werd in januari 1981 "cum laude" het doctoraaldiploma behaald, met als hoofdvak celbiologie-histologie (Prof. F.C.G. v.d. Veerdonk, Utrecht) en als bijvakken embryologie (Prof. N.H. Verdonk, Utrecht), chemische endocrinologie (Prof. H.J. van der Molen, Rotterdam) en didaktiek (Pedagogisch Didaktisch Instituut, Utrecht). Tijdens de studie is verscheidene malen geassisteerd bij zoologie- en histologie-praktika van biologie en diergeneeskunde studenten, en een half jaar gewerkt als "assistent betrokken bij het wetenschappelijk onderzoek" op de afdeling celbiologie-histologie (Prof. v.d. Veerdonk). In de periode van december 1980 tot maart 1981 werd biologieles gegeven op de Christelijke Scholengemeenschap "Oude Hoven" te Gorinchem.

Het in dit proefschrift beschreven werk werd 1 mei 1981 gestart op de afdeling Biochemie II (Chemische Endocrinologie) van de Erasmus Universiteit te Rotterdam. Dit werd mogelijk door een aanstelling als adjunct wetenschappelijk ambtenaar, de eerste drie jaren via FUNGO en vervolgens een half jaar via de universiteit. In deze periode is ook praktikum onderwijs gegeven aan 1e en 2e jaars studenten geneeskunde. In oktober 1985 volgde een aanstelling als wetenschappelijk onderzoeker bij de afdeling Celbiologie I van de Erasmus Universiteit te Rotterdam.

APPENDIX PAPER I

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The steroidogenic activity of isolated Leydig cells from mature rats depends on the isolation procedure

By

R. Molenaar, F. F. G. Rommerts and H. J. van der Molen

Three different collagenase dispersion techniques for isolation of Leydig cells from testes of mature rats have been compared with respect to the yield and quality of the isolated cells. No Leydig cells could be isolated after perfusion of the testis with collagenase (10 mg/ml) followed by incubation at 37°C without shaking, whereas Leydig cells were obtained after incubation of decapsulated testis tissue with collagenase (1 mg/ml) and shaking (80 cycles/min or 1500 cycles/min). Of the total phenylesterase activity, which is localized in the endoplasmic reticulum of interstitial cells, 50 and 85% was released after shaking at 80 and 1500 cycles/, respectively. After sedimentation of the cells 14 and 56% of the microsomal esterase activity was present in the supernatant, whilst 6 and 10% of the esterase activity could be recovered in Ficoll-purified cells. The high esterase activity in the supernatant and the low esterase activity in the purified cells indicated the presence of many broken cells. Testosterone production by cells prepared with the low shaking frequency could be stimulated 9-fold by LH, whereas cells prepared with the high shaking frequency did not respond to LH. Cell preparations which could be stimulated with LH could also be stimulated (3-fold) with NADPH, indicating the presence of damaged cells in addition to intact cells. The percentage of damaged cells in the preparations was estimated using an NADH-dependent histochemical test for mitochondrial diaphorase activity, which shows activity when cell membranes are damaged. Using this criterion, it was found that about 30% of ficoll-purified cells were damaged, whereas only 2% of cells which were pre-incubated and attached to plastic appeared to be damaged. In

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the latter, stimulation of testosterone production by LH was increased and that by NADPH decreased when compared to the original cell suspension.
Isolated interstitial cells from germ cell-depleted rats were subfractionated by density centrifugation. This resulted in a cell fraction enriched in naphtyl-esterase-positive cells and in a fraction containing only a few esterase-positive cells. The former fraction was at least 100-fold more active in terms of steroid production than the latter fraction. Hence, it was concluded that naphtyl-esterase activity can be used as a specific marker for Leydig cells. A quantitative naphtyl-esterase assay, which can be used in both broken and intact cells was developed to determine the number of Leydig cells attached to plastic. The maximal LH-stimulated testosterone production by these cells was 883 ± 465 ng/h/10^6 cells and the stimulation factor was 15.5 ± 4.0.

Key words: Leydig cells – esterase – testosterone production – cell isolation – histochemistry.

Different preparations of Leydig cells from adult rats have been used for studies on the biochemical mechanisms involved in regulation of steroidogenesis by LH. Initially, Leydig cell function was studied in interstitial tissue obtained by wet dissection of testis tissue (Christensen & Mason 1965; Rommerts et al. 1973b). However, when it was found that such tissue showed a 1.5-5-fold variation of the LH-dependent stimulation of testosterone production (Dufau et al. 1971; Rommerts et al. 1973a; Aldred & Cooke 1982; Sharpe & Cooper 1982) this stimulated the development of alternative techniques for isolation of Leydig cells. Leydig cell preparations wich produced more testosterone have been obtained after dispersion of intact testis tissue with collagenase. However, a large variation in LH-stimulated testosterone production, ranging from 3 to 100 ng/10^6 Leydig cells/h and a stimulation factor ranging from 4- to 12-fold is still seen when using this type of preparations (Janszen et al. 1976; Grotjan et al. 1978; Cigorraga et al. 1980; Payne et al. 1980a; Purvis et al. 1980a; Chen et al. 1981; Cooke et al. 1981; Sharpe & Cooper 1982).

Recently, the existence of at least 2 subpopulations of Leydig cells, which differ in the degree of LH-stimulated testosterone production, has been demonstrated (Janszen et al. 1976; Schumacher et al. 1978; Payne et al. 1980a; Chen et al. 1981; Cooke et al. 1981). However, since after collagenase treatment, even the best cell prepatations contained only 10% of the Leydig cells (including damaged cells) present in the total testis tissue (Blankenstein et al. 1976; Janszen et al. 1976; Mori & Christensen 1980), this preparation may not be representative of the total Leydig cell population. The aim of the present study, was therefore to evaluate various procedures used for the isolation of Leydig cells with respect to the yield and quality of cells.

Materials and Methods

Materials

Ovine LH (NIH-LH-S18; 1.03 IU/mg) was a gift from the Endocrinology Study Section of the National Institute of Health, Bethesda, MD, USA. Modified Eagle's minimal essential medium with Earle's salts and non-essential aminoacids was used with additions of 100 µg streptomycin/ml, 100 IU penicillin/ml, 0.6 µg Fungizone/ml and 1% Foedal Calf Serum (Gibco) (Rommerts et al. 1982).

Immature male rats (21 days old) and adult male rats (10 to 14 weeks old) from a Wistar substrain (R-Amsterdam) were used. Germ cell depleted rats were obtained by pre-natal irradiation (150 Rad at day 20 of gestation).

Isolation of cells

Rats were killed in a CO_2 chamber, and the testes were immediately removed. Three different methods for isolation of Leydig cells were used.

A. Testes were perfused via the testicular artery with Krebs Ringer bicarbonate buffer pH 7.4 containing 0.2% glucose (KRBG) and 10 mg collagenase/ml. After decapsulation 2 testes were incubated for 20 min at 37°C, without shaking, in closed plastic incubation tubes containing 10 ml KRBG.

B. Two testes were decapsulated and incubated in closed plastic incubation tubes

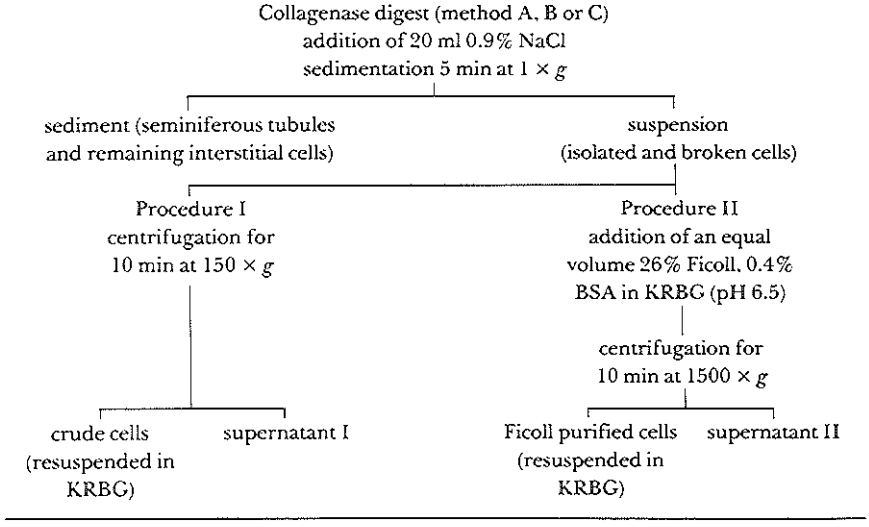

Fig. 1.
Methods used for fractionation of Leydig cells. All steps were performed at room temperature.

containing 10 ml KRBG with 1 mg collagenase/ml. The tubes were shaken longitudinally at approximately 80 cycles/min for 20 min at 37°C.

C. Two testes were decapsulated and incubated in Falcon flasks with 10 ml KRBG and 1 mg collagenase/ml. The flasks were shaken by a flask shaker (Griffin & George Ltd.) at approximately 1500 cycles/min for 20 min at 37°C.

The subsequent isolation of cells was performed essentially as described by Janszen et al. (1976) (see fig. 1).

For subfractionation of Leydig cells, cell suspensions were incubated for 1 h at 37°C in 2 ml culture medium in plastic Petri dishes (Lux, diameter 35 mm). Floating cells were removed by washing and the attached cells were used for further experiments (Rommerts et al. 1982).

Immature Leydig cells were isolated by incubation of 10 decapsulated testes in 10 ml KRBG with collagenase according to method B and fractionated using procedure I in Fig. 1. The method for isolation of tumour Leydig cells has been described previously (Cooke et al. 1979). Testes from germ cell depleted rats were incubated according to method B and fractionated using procedure II in Fig 1. The Ficoll purified cells were enriched in naphtyl estesterase positive cells. After mixing supernatant II with KRBG (3 volumes) and centrifugation for 10 min at 150 × *g*, a sediment enriched in cells without napthyl esterase activity was obtained.

Steroid production by isolated cells

Steroid production was measured either in the medium of total cell suspensions immediately after isolation or in the medium of subfractions attached to plastic dishes (Rommerts et al. 1982). Cells were incubated for 1 h in the precence or absence of LH (100 ng/ml for testis Leydig cells and 1000 ng/ml for tumour Leydig cells) or after adition of an NADPH-generating system (10 mM glucose-6-phophate, 1 mM $NADPH^+$ and 0.3 units glucose-6-phophate dehydrogenase per ml). The amounts of testosterone (for adult Leydig cells) and pregnenolone (for immature and tumour Leydig cells) were measured in the medium by radioimmunoassay as described previously (Rommerts et al. 1982).

Histochemistry

3β-Hydroxysteroid dehydrogenase (3β-HSD) activity. A few drops of staining medium consisting of 5α-androstane-3β-01-17-one (0.003%), Nitro Blue Tetrazolium (0.017%), nicotinamide (0.017%) and NAD (0.017%) in 0.1 M phosphate buffer, pH 7.1, containing 3% Ficoll were added to cells attached to plastic. The cells were frozen in liquid nitrogen, thawed and incubated in staining medium at 37°C for 1 h. The percentage of blue stained cells was estimated.

Naphtyl esterase activity. The percentage naphtyl esterase positive cells was estimated histochemically according to Rommerts et al. (1973b).

In some experiments esterase histochemistry was carried out subsequent to 3β-HSD histochemistry.

Diaphorase activity. Cells were incubated at 37°C in the presence of 0.2 mg Nitro Blue Tetrazolium (NBT)/ml and 0.4 mg NADH/ml. After approx. 15 min the percentage of blue stained cells was estimated.

Enzyme assays

Phenyl esterase activity. Phenyl esterase activity was measured as described by Rommerts et al. 1973b).

Naphtyl esterase activity. 10^3-10^4 Leydig cells (counted in a haemocytometer) were incubated for 2–10 min at room temperature in 1 ml 0.1 M phosphate buffer, containing 1% albumin, pH 7.4, with 0.01% naphtyl acetate as a substrate. The reaction was terminated by addition of 1 ml 2% sodium dodecyl sulphate in 0.1 M phophate buffer. After addition of 2 ml freshly prepared 2 mM fast blue BB the colour was developed during 1 h in the dark. The naphtol production was determined at 3 different concentrations of Leydig cells by measuring the extinction at 540 nm. From this relationship the number of Leydig cells attached to plastic could be calculated from the esterase activity.

Statistical analysis

For statistical analysis Student's *t*-test for paired data was used.

Results

The 3 methods for the isolation of Leydig cells from mature rats were compared with respect to the yield and quality of the cells. For investigation of the distribution and yield of interstitial cells, phenyl esterase has been used as a marker enzyme. LH- and NADPH-dependent testosterone production were used as markers for the quality of Leydig cells.

Distribution of phenyl esterase in isolated fractions

Phenyl esterase activity was measured in fractions obtained using various cell preparation methods.

No interstitial cells were obtained after perfusion of the testis with a high concentration of collagenase and subsequent incubation (method A). A combination of collagenase and shaking resulted in release of esterase activity from the testis. Using method B (80 cycles/min) and method C (1500 cycles/min), about 50% and 80% of the interstitial esterase activity was released, respectively (Table 1). This activity could not be sedimented completely after centrifugation for 10 min at

Table 1.

Phenyl esterase activity in various fractions of rat testes after cell preparation using method B (collagenase and shaking at 80 c/min) or method C (collagenase and shaking at 1500 c/min).

Fraction	Percentage of interstitial activity		Specific activity in µmol/min/mg protein	
	B	C	B	C
Total testis	–	–	0.24 ± 0.01 (4)	0.24 ± 0.01 (4)
(Tubuli and) remaining interstitial cells	52.0 ± 11.9* (3)	11.5 ± 4.5* (4)	0.16 ± 0.04 (4)	0.12 ± 0.02 (3)
Isolated and broken cells	46.9 ± 14.5 (3)	84.5 ± 4.1 (4)	0.27 ± 0.07 (3)	0.25 ± 0.01 (3)
Crude cells	23.3 ± 3.3 (3)	29.7 ± 9.6 (4)	1.15 ± 0.46 (5)	0.10 ± 0.00 (2)
Supernatant I	13.8 ± 2.9 (3)	55.5 ± 5.7 (4)	–	–
Ficoll purified cells	10.1 ± 3.6 (3)	5.6 ± 0.0 (2)	2.07 ± 0.69 (2)	–

* 90% of total testis activity is localized in interstitial tissue. Activity of the remaining interstitial cells was calculated from the total activity after correction for the tubular activity. Means ± SD; number of cell preparations in parentheses.

150 × *g*. For method B, approx. 14% and for method C approx. 56% of the interstitial activity was present in the supernatant. A further purification of the cell preparations, using sedimentation trhough 13% Ficoll, resulted in a yield of esterase activity of about 10% for method B and 6% for method C (Table 1).

The specific esterase activity of the crude cells prepared with method C was lower than in total testis tissue. The purity of crude cells obtained with method B was about 5 times higher and purification by Ficoll resulted in an enrichment of about 9-fold (Table 1).

Treatment with trypsin, dispase or a collagenase-dispase mixture in combination with shaking (80 cycles/min) was also investigated, but did not result in a higher cell yield (results not shown).

Quality of isolated cells

The quality of the cell preparations was investigated by measuring the stimulation of testosterone production by LH (contribution of intact cells) or NADPH (contribution of cells with damaged cell membranes). Since the combination of the effects of LH and NADPH on testosterone production is characteristic for the quality of cell preparations, we have used a 'quality factor', which is defined as the stimulation factor obtained after addition of LH divided by that obtained after addition of NADPH (Table 2).

Crude cells obtained by method B (80 cycles/min) could be stimulated by LH about 9-fold and Ficoll purified cells by about 13-fold, whereas cells obtained by method C (1500 cycles/min) did not respond. The quality factor of cells prepared with method B was the highest and in the following experiments only method B was used. In all cell preparations testosterone production was increased after addition of NADPH (Table 2). The percentage of damaged cells was also estimated using a histochemical NADH-dependent test for intracellular diaphorase activity. The percentage of stained cells (permeable to NADH) was reduced after attachment to plastic and this correlated with an increase in the quality factor (Table 3). However, some diaphorase positive cells were still present among the attached cells and a small effect of NADPH on steroid production was always observed. Pre-incubated and attached Leydig cells from tumour tissue or immature testes did not respond to NADPH and showed a stimulation by LH of approximately 5- and 24-fold, respectively (Table 4).The percentage of diaphorase positive cells in these preparations was $< 0.1\%$.

Naphtyl esterase as a marker for Leydig cells

Ficoll purified cells of mature rats contain about 70% naphtyl esterase positive cells, but it is uncertain if only Leydig cells contain naphtyl esterase activity. Cell fractions enriched in naphtyl esterase positive or negative cells were isolated from germ cell depleted rats. Subfractions obtained after attachment, containing more than 90%

Table 2.
Effect of LH and NADPH on testosterone production of cells, isolated by method B (collagenase, 80 c/min and method C (collagenase, 1500 c/min).

Fraction	Method B			Method C		
	Stimulation factor*		Quality factor**	Stimulation factor*		Quality factor**
	by LH	by NADPH		by LH	by NADPH	
Crude cells	8.6 ± 3.5	2.6 ± 0.4	3.2 ± 1.0	1.0 ± 0.1	3.6 ± 0.7	0.3 ± 0.1
Ficoll purified cells	12.5 ± 6.0	4.0 ± 1.8	4.0 ± 2.5	1.0 ± 0.0	7.0 ± 0.5	0.1 ± 0.0

* Stimulation factor: testerone production with LH/NADPH in ng/h divided basal testosterone production in ng/h.
** Quality factor: stimulation factor by LH divided by stimulation factor by NADPH.
Means ± SD of 2–5 different cell preparations.

Table 3.
Effect of cell attachment to plastic and preincubation on the quality factor of cell preparations and the percentage of diaphorase positive cells.

Criterion for quality	Crude cells		Ficoll purified cells	
	Total	Attached to plastic	Total	Attached to plastic
Quality factor	3.2 ± 1.0* (3)	7.2 ± 2.2* (3)	4.0 ± 2.5* (5)	8.7 ± 3.6* (5)
Percentage diaphorase positive cells	17.5 ± 6.5**(4)	3.8 ± 4.8**(4)	27.8 ± 3.2**(4)	2.0 ± 2.3**(4)

Significant increase after attachment: * $P < 0.05$
Significant decrease after attachment: ** $P < 0.005$
Means ± SD; number of cell preparations in parentheses.

positive cells (Ficoll pellet fraction) responded to LH, whereas fractions containing less than 5% esterase positive cells (Ficoll supernatant fraction) produced only small amounts of testosterone. The effect of NADPH on testosterone production was small for both fractions (Table 5). The distribution of 3β-HSD positive cells was similar to that observed for esterase positive cells. In Leydig cell preparations from normal rats more than 90% of the esterase positive cells also stained positively for 3β-HSD.

Table 4.
Effect of LH and NADPH on steroid production by different preparations of Leydig cells after preincubation (1h) and attachment to plastic.

Tissue	Stimulation factor*	
	LH	NADPH
Tumour	5.1 ± 0.9 (3)	1.2 ± 0.4 (4)
Testes		
immature rats	23.8 ± 2.8 (4)	1.1 ± 0.6 (3)
mature rats	15.5 ± 4.0 (5)	2.1 ± 1.1 (5)

* Stimulation factor: steroid production with LH/NADPH divided by basal steroid production. Means ± SD; number of cell preparations in parentheses.

Table 5.
Naphtyl esterase (N.E.) activity and testosterone (T) production stimulated by LH ór NADPH.

Cell fraction	% age N.E. positive	with LH ng T/10^6 cells/h	with NADPH ng T/10^6 cells/h
Ficoll pellet fraction	> 90	1433 ± 1176 (3)*	108 ± 39 (3)
Ficoll supernatant fraction	< 5	10 ± 9 (3)	2 ± 1 (3)

* LH-stimulation factor 23.0 ± 13.1. Means ± SD; number of cell preparations in parentheses.

A quantitative assay for naphtyl esterase activity in broken and intact cells was developed. The naphtyl esterase activity of 3 different dilutions of Ficoll-purified cells in suspension (estimated by cell counting) was measured and the relationship between the number of cells and esterase activity established. From this relationship the number of attached cells could be calculated after measuring naphtyl esterase activity in these cells.

Since considerable variation in enzyme activity was found for different preparations of Leydig cells (300–1200 nmol/min/10^6cells), the relationship between number of cells and esterase activity was determined in each individual cell preparation. In all of these a linear relationship between number of Leydig cells and esterase activity, and between reaction time and esterase activity was found (Fig. 2).

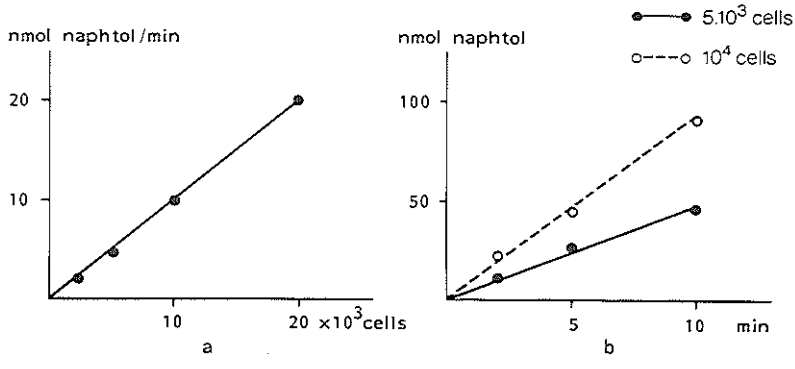

Fig. 2.
Naphtol production of Leydig cells as a function of number of cells (a) and of reaction time (b).

Testosterone production by Ficoll purified Leydig cells

Testosterone production by Ficoll purified Leydig cells was measured using either total cell suspensions or attached cells, incubated either without LH or in the prescence of a maximally stimulating dose of LH. The number of Leydig cells was calculated from the naphtyl esterase activity. Basal and LH-stimulated testosterone production in total cell suspensions were 12 ± 4 (n = 6) and 171 ± 117 (n = 6) ng/h/10^6 Leydig cells, respectively, and in attached cells 79 ± 46 (n = 4) and 883 ± 456 (n = 4) ng/h/10^6 Leydig cells, respectively, (means $\pm$ SD).

Discussion

Three procedures for the isolation of mature rat Leydig cells have been investigated with respect to the yield and quality of the isolated cells.

After perfusion of testes with a high concentration of collagenase and subsequent incubation without shaking, no Leydig cells were released from the testis tissue. Isolated Leydig cells could, however, be obtained by methods employing 10-times less collagenase and shaking. We have, therefore, concluded that mechanical forces are required for the isolation of Leydig cells. Phenyl esterase, which is localized in the endoplasmic reticulum of interstitial cells (Rommerts et al. 1973b; Van der Vusse et al. 1973), has been detected in the supernatant of centrifuged cells, indicating that a portion of the interstitial cells were disrupted. Esterase activity in the remaining tissue indicated that 10% (method B) or 50% (method C) of the interstitial cells were not released, whereas with either method a maximum of 10% of the Leydig cells could be isolated. A low shaking frequency of 80 cycles/min gave the best results, whilst incubation with a higher shaking frequency resulted in a decrease of the esterase content after centrifugation, perhaps reflecting preferential release of non Leydig cells, and/or significant cell damage.

Stimulation of testosterone production by LH requires intact cells, whereas NADPH can stimulate cells with damaged membranes (Tsang & Stachenko 1970; Goverde et al. 1980). The cell preparations were, therefore, characterized by the ratio of the stimulation factor by LH, divided by the stimulation factor by NADPH, thus providing a 'quality factor'. Intact cells with a high stimulation factor by LH and a low NADPH response show a high quality factor, whereas the factor for damaged cells is low. The quality factor for cell preparations obtained with a high shaking frequency was more than 10 times lower than that obtained after shaking at 80 cycles/min.

The percentage of damaged cells can also be estimated histochemically, using an NADH-dependent reduction of nitro blue tetrazolium salts (NBT). In the absence of intact cell membranes NBT can be reduced by intracellular diaphorase activity, resulting in a blue staining. Cell preparations with the highest quality factor show the lowest NBT-staining. Using the quality factor, the diaphorase test and the

absolute testosterone production per Leydig cell as criteria for cell viability, it appears that the highest percentage of intact cells can be selected from Ficoll-purified cells by pre-incubation and attachment to plastic.

The LH-stimulated testosterone production of pre-incubated and attached Ficoll purified cells (ranging from 400 to 1200 ng/10^6 Leydig cells/h) was 10 to 100 times higher than the production reported by other authors (varying from 3–100 ng/10^6 Leydig cells/h) (Janszen et al. 1976; Grotjan et al. 1978; Cigorraga et al. 1980; Payne et al. 1980a; Purvis et al. 1980; Chen et al. 1981; Cooke et al. 1981; Sharpe & Cooper 1982). This can be partly explained by removal of cells with damaged membranes by washing the dishes. We observed a correlation between esterase activity, 3β-HSD activity and testosterone production, and similar results were obtained by Kühn-Velten et al. (1982). Furthermore, Leydig cells characterized by electron microscopy also showed esterase activity, whereas cells which were not clearly identified showed no activity (Vorstenbosch, personal communication). These observations indicate that naphtyl esterase activity can be used as a marker for Leydig cells. We prefer naphtyl esterase as marker enzyme to 3β-HSD, since it is 1000-fold more active than 3β-hydroxysteroid dehydrogenase (Van der Vusse et al. 1974) and can be determined quantitatively and qualitatively in intact cells. Hence, we have concluded that naphtyl esterase activity is a better marker enzyme for intact Leydig cells than 3β-hydroxysteroid dehydrogenase.

The heterogeneity of isolated Leydig cells from mature rats, as reported by Payne et al. 1980, Chen et al. 1981 and Cooke et al. 1981, may be partly attributable to cell damage as a result of the isolation procedure. On the other hand, other observations support the existence of functionally heterogeneous Leydig cells, such as cells containing LH receptors without detectable 3β-hydroxysteroid dehydrogenase activity (Purvis et al. 1980b). Effects of hormones (Payne et al. 1980b) and maturation (Chase & Payne 1981) on the distribution and activities of subpopulations have also been observed. Furthermore, functional heterogeneity of Sertoli cells depending on the stage of the spermatogenic cycle has been reported (Parvinen et al. 1980; Lacroix et al. 1981; Gordeladze et al. 1982). Local effects of secretion products from Sertoli cells on Leydig cells have also been suggested (Aoki & Fawcett 1978; Sharpe et al. 1981; Bergh et al. 1982). However, functional heterogeneity can be investigated only in cells which reflect quantitatively and qualitatively the in vivo situation and artifacts caused by the cell preparation technique must be eliminated. Since these conditions have not yet been met, it is unclear to what extent the observed heterogeneity of isolated rat Leydig cells is functional.

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APPENDIX PAPER II

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Non-specific esterase: a specific and useful marker enzyme for Leydig cells from mature rats

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ABSTRACT

The presence of non-specific esterase activity is correlated with different Leydig cell characteristics: 3β-hydroxysteroid dehydrogenase (3β-HSD), human chorionic gonadotrophin binding and LH-stimulated steroid production. This indicates that esterase can be used as a marker enzyme for Leydig cells. Esterase, however, has also been used as a marker enzyme for macrophages. We have compared, using biochemical and histochemical techniques, the esterase activity of Leydig cell preparations from mature and immature rats and of preparations enriched in testicular or peritoneal macrophages. Leydig cells were identified by staining for 3β-HSD, and macrophages by phagocytosis of fluorescent beads. Leydig cell preparations from mature rats showed an approximately 400-fold higher esterase activity than peritoneal macrophage preparations and an approximately 50-fold higher activity than testicular macrophage preparations. Leydig cell preparations from mature rats showed a 60-fold higher esterase activity than Leydig cell preparations from immature rats.

Differences in esterase activity were also demonstrated histochemically. Leydig cells from mature rats showed positive esterase staining after 30 s at room temperature. Testicular macrophages showed esterase activity after staining for 3 min. Only approximately 25% of the 3β-HSD-positive cells from immature rats showed esterase activity after staining for 6 min. Esterase is therefore a useful marker enzyme for Leydig cells from mature rats and can be of help in studies concerning the development of these cells.
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INTRODUCTION

Non-specific esterase activity is localized in the interstitial tissue in the testes of mature rats (Niemi, Härkönen & Ikonen, 1966; Rommerts, van Doorn, Galjaard *et al.* 1973; van der Vusse, Kalkman & van der Molen, 1973). Several authors have demonstrated, using isolated cells from mature rats, that this esterase activity is correlated with 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, human chorionic gonadotrophin (hCG) binding and luteinizing hormone-stimulated steroid production (van der Vusse *et al.* 1973; Janszen, Cooke, van Driel & van der Molen, 1976; Cooke, Magee-Brown, Golding & Dix, 1981; Kühn-Velten, Wolff, Passia & Staib, 1982). Niemi *et al.* (1966) showed that hypophysectomy caused a clear decrease in esterase activity in the interstitial tissue. These results indicate that esterase is localized in the Leydig cells and may be used as a Leydig cell marker. Generally, 3β-HSD is used as a marker enzyme to estimate the percentage of Leydig cells in cell preparations. The staining procedure for 3β-HSD activity requires that the cell membranes are made permeable for NAD^+. Since damage of the cell membrane may also result in damage or release of the enzyme, the original distribution of 3β-HSD activity may be disturbed. In contrast, staining for non-specific esterase activity can be performed on intact cells and may therefore be an easier and more reliable method for the characterization of Leydig cells than staining for 3β-HSD activity. Furthermore, during maturation a clear increase in the histochemical staining of non-specific esterase of the interstitium (Niemi & Ikonen, 1963), and in the specific activity of total testis tissue (Meyer, Forsgren, von Deimling & Engel, 1974) has been observed. Also, Meyer *et al.* (1974) showed a shift in the pattern of isoenzymes during maturation which could be enhanced by treatment of immature animals with hCG. These results suggest that esterase may be a useful histochemical marker for developing Leydig cells during sexual maturation.

Non-specific esterase has also been used as a marker

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enzyme for macrophages (van Furth & Diesselhoff-den Dulk, 1982; Ennist & Jones, 1983; Onozaki, Takenawa, Homma & Hashimoto, 1983), and is present in testicular macrophages (Yee & Hutson, 1983). The interstitium of the testes of mature rats contains, in addition to Leydig cells, approximately 15–20% macrophages (Christensen, 1975; Dym & Madwah Raj, 1977; Yee & Hutson, 1983). If macrophages contribute to the staining for esterase activity, the use of esterase as a marker for Leydig cells may result in an over-estimation of the numbers of Leydig cells in isolated interstitial cell preparations.

The usefulness of esterase as a marker enzyme for Leydig cells will therefore depend on the relative enzyme activities in the various cell types to be stained. We have, therefore, compared the esterase activity in Leydig cells from mature and immature rats and in testicular and peritoneal macrophages, using biochemical and histochemical techniques.

MATERIALS AND METHODS

Animals

Immature (22 days old) and adult male rats (60–90 days old) from a Wistar sub-strain (R-Amsterdam) were used. Leydig cell-depleted rats were obtained by treating mature rats with ethane dimethyl sulphonate (EDS; 75 mg/kg body weight) 3 days before cell isolation, as described previously (Molenaar, de Rooij, Rommerts *et al.* 1985). Labelling with trypan blue showed that macrophages were still present in the interstitium after treatment with EDS (Molenaar *et al.* 1985).

Isolation of cells

Enriched Leydig cell preparations from the testes of mature and immature rats were obtained as described by Rommerts, Molenaar & van der Molen (1985). Enriched testicular macrophage preparations were obtained from the testes of Leydig cell-depleted rats using the same isolation procedure as for Leydig cells (Rommerts *et al.* 1985). Peritoneal macrophages were obtained by lavage of the peritoneal cavity of mature rats with 11·6% sucrose, 72 h after one i.p. injection of 15 ml thioglycollate solution (40·5 g/l water).

Characterization of Leydig cells and macrophages

Cells ($1–2 \times 10^6$) were added to plastic Petri dishes (Lux; diameter 3 cm; Lux; Miles Laboratories Inc., Naperville, U.S.A.) containing 2 ml Minimal Essential Medium (containing fugizone (600 ng/ml), streptomycin (100 μg/ml) and penicillin (100 i.u./ml)) and 1% fetal calf serum (Gibco, Breda, The Netherlands). The cells were allowed to attach to the plastic during a 1-h incubation at 37 °C.

Leydig cells were identified by staining for 3β-HSD activity as described previously (Molenaar, Rommerts & van der Molen, 1983). Briefly, cells attached to the Petri dish were covered by a drop of staining medium containing 5α-androstane-3β-ol-17-one, nitro-blue tetrazolium, nicotinamide and NAD^+ (pH 7·1). They were then frozen in liquid nitrogen and subsequently thawed and incubated in 1 ml staining medium for 1 h at 37 °C.

Macrophages were identified by their phagocytic activity. Phagocytic activity which was demonstrated *in vivo* by labelling with trypan blue (Molenaar *et al.* 1985) was evaluated *in vitro* by phagocytosis of fluorescent beads. This was determined by incubation of cells with fluorescent beads (diameter 1 μm; Polysciences Inc., Warrington, PA, U.S.A.) for 2 h at 37 °C, followed by extensive washing. An overlap of more than 95% between the results obtained from labelling *in vivo* with trypan blue and those obtained *in vitro* with fluorescent beads was observed, demonstrating the validity of this latter procedure. After washing no overlap between positive staining for 3β-HSD activity and the presence of fluorescent beads was observed.

Esterase activity in isolated cell preparations was measured by nitrophenol production from p-nitrophenylacetate as described by Rommerts *et al.* (1973).

The histochemical estimation of α-naphthyl esterase (α-NE) activity in cells attached to plastic was carried out as described by Rommerts *et al.* (1985). Briefly, the staining medium, containing α-NE, $NaNO_2$ and pararosaniline–HCl (pH 8·4), was prepared freshly and kept in the dark at 0 °C. Intact cells were incubated at room temperature for 30s (Leydig cells from mature rats) or 3–6 min (macrophages and Leydig cells from immature rats).

RESULTS

Phenyl esterase activity in Leydig cell-enriched preparations from mature and immature rats and in preparations enriched in testicular and peritoneal macrophages from mature rats are shown in Table 1, as are the percentages of 3β-HSD-positive cells (Leydig cells) and phagocytic cells (macrophages).

In Leydig cell preparations from mature rats, cells were present which were not actively phagocytotic but which showed esterase activity after staining for 30 s (Plate, fig. 1*a* and *b*). In testicular macrophage preparations, containing many phagocytic cells, no esterase-positive cells were observed after staining for 30 s (Plate, fig. 1*c* and *d*), but after staining for 3 min the phagocytic cells showed esterase activity (Plate, fig. 1*e* and *f*). Furthermore, in Leydig cell preparations

TABLE 1. Production of p-nitrophenol from p-nitrophenylacetate by preparations containing rat Leydig cells (3β-hydroxysteroid dehydrogenase (3β-HSD)-positive) or macrophages (actively phagocytotic). Values are either the range or the mean ± S.D.; numbers of cell preparations are shown in parentheses

	3β-HSD-positive cells (% of total)	Phagocytic cells (% of total)	p-Nitrophenol (nmol min per 10^6 nucleated cells)
Preparation			
Leydig cells (mature rats)	80 ± 7 (5)	10–15 (3)	407·3 ± 99·4 (6)
Leydig cells (immature rats)	43 ± 7 (5)	< 1 (4)	6·1 ± 1·8 (3)
Testicular macrophages (mature rats)	0 (4)	30–40 (3)	8·6 ± 1·1 (4)
Peritoneal macrophages (mature rats)	0 (4)	> 90 (4)	1·2 ± 0·1 (3)

from mature rats an overlap was observed in more than 95% of the cells between 3β-HSD activity and esterase activity (staining for 30 s; data not shown). These data indicate that Leydig cells can be stained selectively for esterase activity after incubation for 30 s. Thus, in the presence of Leydig cells, macrophages can be identified by their phagocytic activity and not by staining for esterase activity, since Leydig cells have much greater esterase activity but do not show phagocytic activity.

Leydig cell preparations from mature rats showed an approximately 400-fold higher esterase activity than peritoneal macrophage preparations, and an approximately 50-fold higher activity than testicular macrophage preparations (Table 1). Leydig cell preparations from mature rats showed an approximately 60-fold higher esterase activity than Leydig cell preparations from immature rats. Testicular macrophage preparations, containing 30–40% macrophages and no Leydig cells, had a sevenfold higher esterase activity than peritoneal macrophages (more than 90% pure).

Leydig cell preparations from immature rats, containing 3β-HSD-positive cells (Plate, fig. 2*a*), showed no esterase activity after staining for 1 min (Plate, fig. 2*b*), but after staining for 6 min, esterase-positive cells were present in the preparation (Plate, fig. 2*c*). The cells which showed esterase activity always showed 3β-HSD activity (data not shown); however, only approximately 25% of the 3β-HSD-positive cells showed histochemical esterase activity.

A comparison of the esterase activity in the interstitial tissue in sections of whole testis obtained from normal mature rats and from Leydig cell-depleted rats showed a clear staining of the interstitium in normal rats after 30 s (Plate, fig. 3*a*), probably reflecting the presence of Leydig cells, and a more intense staining after 3 min (Plate, fig. 3*c*). The interstitium of Leydig cell-depleted rats showed no staining after incubation for 30 s (Plate, fig. 3*b*), but after staining for 3 min a few esterase-positive cells, probably representing the macrophages, could be seen (Plate, fig. 3*d*).

DISCUSSION

Non-specific esterase is generally used as a marker for macrophages (van Furth & Diesselhoff-den Dulk, 1982; Ennist & Jones, 1983; Onozaki *et al.* 1983; Yee & Hutson, 1983), but there are also indications that this enzyme may be used as a marker for Leydig cells (van der Vusse *et al.* 1973; Janszen *et al.* 1976; Cooke *et al.* 1981; Kühn-Velten *et al.* 1982). In the present study we have compared the esterase activity of different Leydig cell and macrophage preparations. Leydig cell preparations were obtained from intact mature and immature rats, whereas preparations enriched in testicular macrophages were obtained from mature rats which were pretreated with EDS. It has been demonstrated that Leydig cells completely disappear from the interstitium 3 days after treatment with EDS, whereas macrophages survive the EDS treatment (Kerr, Donachie & Rommerts, 1985; Molenaar *et al.* 1985; Morris, Phillips & Bardin, 1985). Isolation of interstitial cells from these rats resulted in preparations free of Leydig cells, but enriched in testicular macrophages. The results show that the esterase activity in Leydig cells from mature rats is much higher than the activity in peritoneal and testicular macrophages or in Leydig cells from immature rats.

The esterase activity in Leydig cell preparations from mature rats was approximately 50-fold higher than in testicular macrophage preparations. Moreover, the histochemical data showed that this esterase activity was correlated with 3β-HSD activity and not with phagocytic activity, and that in testis sections as

well as in isolated cell preparations from Leydig cell-depleted rats these esterase-positive cells were absent. We conclude, therefore, that esterase can be used as a marker enzyme for Leydig cells from mature rats. The esterase activity in testicular and peritoneal macrophages was much lower. After correction for the impurity of the preparations, esterase activity in testicular macrophages appeared to be approximately 20 times higher than the activity in peritoneal macrophages. This indicates that the properties of testicular macrophages may be different from those of peritoneal macrophages, which agrees with the current opinion that functional specialization of macrophages takes place in tissues (van Furth, 1981).

In interstitial cell preparations from immature rats the highest esterase activity was present in Leydig cells, but the mean activity/Leydig cell was approximately 30-fold lower than the activity in Leydig cells from mature rats. Since esterase activity in Leydig cells from immature rats is relatively low, this enzyme cannot be used for histochemical discrimination between macrophages and Leydig cells in preparations from immature rats. The observed increase in esterase activity/Leydig cell during testicular development agrees with the observations of Meyer *et al.* (1974) who found an approximately 40-fold increase in esterase activity/gram testis tissue between days 20 and 60 after birth. The amount of Leydig cells/gram testis, however, is almost constant over this period (Tapanainen, Kuopio, Pelliniemi & Huhtaniemi, 1984), and Niemi & Ikonen (1963) found a clear increase in the percentage of esterase-positive areas from day 18 onwards. This increase in esterase activity is correlated with a shift in the pattern of esterase isoenzymes (Meyer *et al.* 1974). Only some of the 3β-HSD-positive (Leydig) cells from immature rats showed histochemical esterase activity, whereas all those from mature rats showed a high esterase activity in the present study. This suggests that the development of esterase in Leydig cells does not occur in all cells at the same time. Esterase activity may thus be a useful means of measuring the maturation of Leydig cells.

Activity of 3β-HSD is generally accepted as a marker for Leydig cells, but there are some arguments in favour of the use of esterase as a marker for Leydig cells from mature rats. The esterase activity in the interstitium, which is localized in the endoplasmic reticulum, is approximately 100-fold higher than the 3β-HSD activity (van der Vusse *et al.* 1973). Furthermore, esterase activity can be determined qualitatively and quantitatively using simple methods, and a good correlation between these methods has been shown in this paper. The quantitative assay has already proved useful in previous studies concerning the isolation of Leydig cells, which have led to the conclusion that approximately 50% of the Leydig cells are disrupted during standard isolation procedures, since esterase activity was detected in the cell-free supernatant (Molenaar *et al.* 1983). Moreover, staining for esterase activity can be performed with intact cells, in contrast with the staining for 3β-HSD activity which requires a step which makes the cell membrane permeable for NAD^+, e.g. drying at room temperature (Payne, Downing & Wong, 1980), freezing at −15 °C (Cooke *et al.* 1981) or freezing in liquid nitrogen (Molenaar *et al.* 1983). Damage of the cell membrane, however, may lead to disruption of cells causing deterioration of cell morphology, decreased enzyme activity or detachment of cells from plastic surfaces. Some authors (Mendelson, Dufau & Catt, 1975; Browning, d'Agata & Grotjan, 1981) have applied 3β-HSD histochemistry directly on isolated cells, without a step to make the cell membrane permeable, and have then estimated the numbers of stained cells. Under such conditions the presence of extracellular 3β-HSD, as demonstrated for esterase (Molenaar *et al.* 1983), accounts for the staining. Since the enzyme reactions take place in the medium and not within the cells, the precipitate formed will show a random distribution on almost all cells and an erroneously high percentage of 'Leydig cells' will be measured. These difficulties in estimating the true number of Leydig cells may have contributed to the large variation in rates of testosterone production, ranging from 3 to 100 ng/10^6 Leydig cells per hour reported by different authors (Janszen *et al.* 1976; Grotjan, Tcholakian & Steinberger, 1978; Cigorraga, Sorrell, Bator *et al.* 1980; Payne *et al.* 1980; Purvis, Clausen & Hansson, 1980; Chen, Lin, Murono *et al.* 1981; Cooke *et al.* 1981).

Using biochemical techniques we have shown that Leydig cells from mature rats contain a much higher esterase activity than macrophages and that this difference in activity can be used for a histochemical identification of Leydig cells. The incubation time for developing the stain is, however, of paramount importance, since esterase is a very active enzyme. Leydig cells from mature rats can be identified by staining for 30 s at room temperature. When the incubation is continued for 3 min, however, macrophages also appear positive and existing differences in esterase activities can be masked. This may result in an over-estimation of the number of Leydig cells. Furthermore, the differences in the biochemically estimated esterase activities between the different cell types are not directly reflected by the various incubation times required for positive staining. Hence, a comparison of the staining intensities after histochemistry only gives a semi-quantitative impression of the enzyme activities of the different cells.

When correct incubation times are used, non-specific esterase is a specific and useful marker enzyme for Leydig cells from mature rats, because it is a very

active enzyme which can be estimated with a simple histochemical and biochemical assay. Moreover, the non-specific esterase activity can be used as a marker in the study of the development of Leydig cells.

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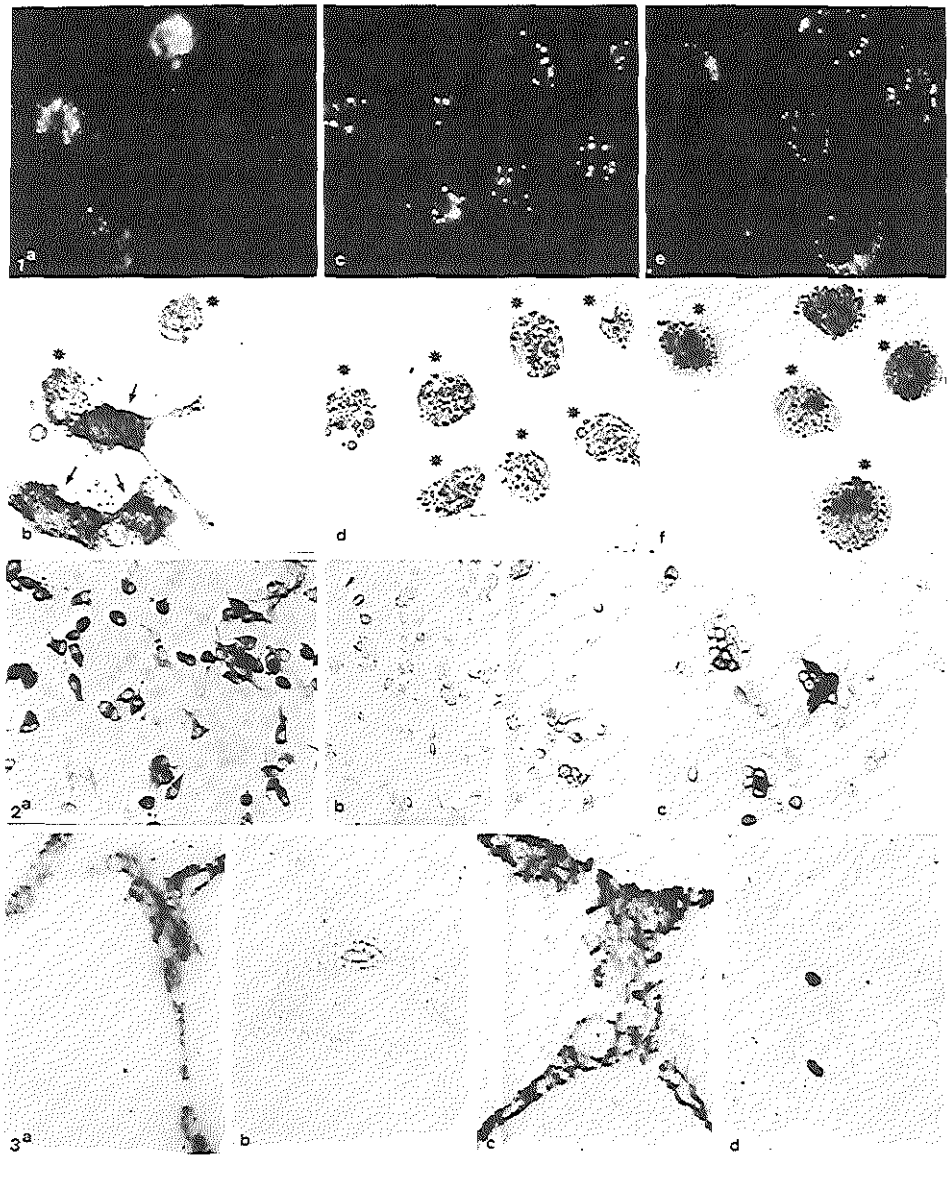
DESCRIPTION OF PLATE

FIGURE 1. Interstitial cell preparations from normal and Leydig cell-depleted (LCD) mature rats. (*a*, *c* and *e*) Cells were allowed to phagocytose fluorescent beads for 2 h *in vitro* and, after washing, this was followed by (*b*, *d* and *f*) staining for α-naphthyl esterase (α-NE) activity. (*a* and *b*) Interstitial cell preparation from normal mature rats containing Leydig cells (arrowed) and macrophages (asterisks). Staining for α-NE activity was performed for 30 s. (*c*, *d*, *e* and *f*) Testicular macrophage preparations from LCD rats containing macrophages (asterisks) and no Leydig cells. Staining for α-NE activity was performed for (*c* and *d*) 30 s and (*e* and *f*) 3 min (× 500).

FIGURE 2. Interstitial cell preparations from immature rats. Staining for (*a*) 3β hydroxysteroid dehydrogenase activity for 1 h, (*b*) α-NE activity for 1 min and (*c*) α-NE activity for 6 min (× 180).

FIGURE 3. Testis sections from (*a* and *c*) normal mature rats and (*b* and *d*) LCD rats stained for α-NE activity for (*a* and *b*) 30 s and (*c* and *d*) 3 min (× 180).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APPENDIX PAPER III

Biol. Reprod. 33 (1985), 1213-1222

BIOLOGY OF REPRODUCTION 33, 1213–1222 (1985)

Specific Destruction of Leydig Cells in Mature Rats after In Vivo Administration of Ethane Dimethyl Sulfonate

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ABSTRACT

Effects of ethane dimethyl sulfonate (EDS) on Leydig cells have been studied using the following parameters: morphology, histochemistry of 3β-hydroxysteroid dehydrogenase (3β-HSD) and esterase, quantitative activity of esterase, testosterone concentrations in plasma, and steroid production by isolated interstitial cells in vitro. Degenerating Leydig cells were observed within 16 h after the injection of mature rats with EDS (75 mg/kg body weight). At that time the testosterone concentration in plasma and the specific activity of esterase in testis tissue were decreased to approximately 35% and 60% of the control value, respectively. At 48 h after EDS only a few normal Leydig cells were left and the plasma testosterone concentration was less than 5% of the control value. The specific activity of esterase in total testis tissue was similar to the activity of dissected tubules from untreated rats. At 72 h no Leydig cells could be detected and no 3β-HSD and esterase-positive cells were present. At that time macrophages were still present in the interstitium and the appearance of the spermatogenic epithelium was normal, but 1 wk after EDS the elongation of spermatids was disturbed, probably due to a lack of testosterone. In some of the animals the cytotoxic effects of EDS on Leydig cells could be partly inhibited by human chorionic gonadotropin treatment. The basal steroid production by interstitial cells from mature rats 72 h after EDS was not significant and no stimulation by LH was observed, whereas no effect of EDS could be detected on steroid production by interstitial cells isolated from immature rats and mice 72 h after treatment. Other compounds with similar structures, such as butane dimethyl sulfonate (busulfan) and ethane methyl sulfonate (EMS) had no effect on Leydig cells from mature rats. It is concluded that EDS specifically destroys Leydig cells in mature rats.

INTRODUCTION

Diesters of methane sulfonic acid [general formula: $CH_3OSO_2(CH_2)_nCH_3OSO_2$] are well known to affect fertility in the male. Methane dimethyl sulfonate (MDS, n=1), propane dimethyl sulfonate (PDS, n=3), and butane dimethyl sulfonate [busulfan (Myleran), n=4] specifically injure the spermatogonia (Cooper and Jackson, 1970; de Rooij and Kramer, 1970; van Keulen and de Rooij, 1974). Ethane dimethyl sulfonate (EDS, n=2) also damages the spermatogenic epithelium, but in addition causes a decrease in androgen levels with a resulting loss of weight of the ventral prostate, seminal vesicles, and epididymis (Jackson and Morris, 1977; Morris and McCluckie, 1979; Jackson and Jackson, 1984). The effect of EDS on the spermatogenic epithelium and the accessory organs could be prevented by simultaneous administration of testosterone (Jackson and Jackson, 1984). These observations have led to the hypothesis that EDS, in contrast to the other sulfonic esters, primarily injures the Leydig cells and that the reduction of local testosterone levels causes degenerative pro-

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cesses in the germinal epithelium. It has been found (Jackson and Morris, 1977; Morris and McCluckie, 1979) that spermatogenesis and a normal appearance of the accessory organs are restored approximately 7 wk after administration of EDS. These observations show that ultimately the effects of EDS are reversible. This may indicate either that EDS causes a temporary loss in activity of the Leydig cells present, without actually destroying the cells, or that Leydig cells are destroyed and that regeneration occurs.

We have therefore investigated whether EDS inhibits Leydig cell function temporarily or destroys Leydig cells, using the following parameters: morphology of the Leydig cells, activity of the marker enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and esterase, and steroid production in vivo and in vitro.

MATERIALS AND METHODS

Materials

EDS, which is not commercially available, was synthesized as described by Jackson and Jackson (1984) from ethylene glycol and methane sulfylchloride. After recrystallization from dichloromethane twice, the EDS was more than 99% pure as checked by nuclear magnetic resonance. Busulfan and ethane methyl sulfonate (EMS) were purchased from Burroughs Wellcome and Co. and K & K Chemicals, respectively. Human chorionic gonadotropin (hCG; Pregnyl) was obtained from Organon, Oss.

Treatment of Animals

Adult (2–3-mo-old) and immature (22-day-old) rats from a Wistar substrain R-Amsterdam and mature Swiss mice (approx. 3 mo old) were used.

EDS [30 mg/ml in dimethyl sulfoxide (DMSO): H_2O, 1:3] was administered in one intraperitoneal (i.p.) injection (75 mg/kg body weight). As a control a single i.p. injection of the vehicle was given. Busulfan (suspended in arachis oil) and EMS (dissolved in DMSO:H_2O, 1:3) were given i.p. in equimolar concentrations (i.e., 85 mg and 43 mg/kg body weight, respectively). In some experiments macrophages were labeled by one i.p. injection with 3% trypan blue in saline (1 ml/100 g body weight) 3 days before the death of the animal (Christensen and Peacock, 1980). Human chorionic gonadotropin (100 IU) was administered on 3 consecutive days preceding the EDS injection by subcutaneous injection and on Day 4 hCG was given in combination with EDS.

Measurements

Animals were killed by decapitation. Blood was collected and plasma was stored at −20°C. Testes were removed immediately. For histology, testis tissue was fixed in Bouin's fluid and 5 μm paraffin and butoxy ethanol-glycol methacrylate sections were made and stained by the periodic acid-Schiff technique and hematoxylin. For enzyme histochemistry, testis tissue was frozen in liquid nitrogen and stored at −80°C. 3β-Hydroxysteroid dehydrogenase and α-naphthyl esterase (α-NE) histochemistry was performed on 10-μm frozen sections according to Loyda et al. (1980) and Rommerts et al. (1973), respectively. Control sections incubated in the absence of substrate or NAD^+ showed no staining. For the quantitative assay of esterase, testis tissue was sonicated and stored at −20°C. Phenyl esterase activity was measured as described by Rommerts et al. (1973).

Interstitial cells were isolated from decapsulated testes by treatment with collagenase (rats) as described by Janszen et al. (1976) and Rommerts et al. (1982) or dissociated mechanically (mice) as described by Schumacher et al. (1978).

Testosterone was measured in plasma by radioimmunoassay according to Verjans et al. (1973). Testosterone and pregnenolone were measured in media according to Rommerts et al. (1982).

For statistical analysis, Student's *t*-test for unpaired data was used.

RESULTS

Morphology and Histochemistry

Histologic sections from testes obtained at different intervals after EDS treatment are shown in Fig. 1. As soon as 16 h after injection of EDS many degenerating Leydig cells, showing pycnotic nuclei, were seen (Fig. 1b). However, some of the Leydig cells still looked normal after this interval. After 48 h only a few Leydig cells with a normal appearance remained together with some degenerating Leydig cells (Fig. 1c). After 72 h Leydig cells had become rare (Fig. 1d) and staining for 3β-HSD and α-NE activity did not show any 3β-HSD- or α-NE-positive cells, in contrast to the clear staining of the interstitium in DMSO-treated controls (Fig. 2). No damaging effect of EDS on macrophages was observed (Fig. 1d). After 1 wk Leydig-type cells were absent (Fig. 1f).

During the first 3 days after treatment with EDS no effect was seen on the seminiferous epithelium. However, after 1 wk degenerating pachytene spermatocytes in stages VII and VIII and especially step 9–11 spermatids in stages IX–XI were seen. Furthermore, in stages VII–XII degenerating step 19 spermatids were frequently observed, mainly in the proximity of the basement membrane (Fig. 1e). In most of the tubular cross-sections in stages X–XII the number of step 10–12 spermatids seemed to be reduced, and occasionally these cells were entirely absent, probably as a result of the degeneration of the elongating spermatids (Fig. 1f). In control animals degenerating cells were rare.

Quantitative Esterase Assay

The rate of destruction of Leydig cells by EDS was evaluated quantitatively by measuring phenyl esterase activity in testis tissue (Table 1). The specific esterase activity was decreased to approximately 70%, 10%, and 7% of the original activity at 16, 48, and 72 h after injection, respectively. Esterase activity in testis tissue at 48 and 72 h after EDS was even lower than the activity present in dissected tubules from normal testes, indicating a total loss of Leydig cell activity. Treatment with the DMSO-containing solvent had no effect.

Steroidogenic Activity

Plasma testosterone levels were measured at 16, 48, and 72 h after treatment (Table 2). In spite of the large variation in testosterone levels among control animals, a significant decrease was already observed at 16 h after EDS. At 48 and 72 h after EDS a more than 10-fold decrease in testosterone levels was observed.

The effects of EDS on Leydig cells were also studied by measuring the steroidogenic capacity in vitro of interstitial cell preparations isolated from testes of EDS-treated mature rats (Table 3). The basal pregnenolone production by interstitial cells was not significant and no stimulation by LH was observed.

Specificity of EDS Action

The effects of the related compounds busulfan and EMS on testicular esterase activity and Leydig cell morphology were studied in mature rats 72 h after administration. No effect of these compounds on phenyl esterase activity was observed (Table 4) and the appearance of the Leydig cells and seminiferous tubules was normal (Fig. 3a).

No effect of EMS on the seminiferous epithelium was observed. However, 3 days after injection of busulfan the number of A spermatogonia in stages X–V was reduced as well as the number of In spermatogonia in stages III and V and B spermatogonia in stage V. Many tubular cross-sections in stages X–V showed only one or two spermatogonia or none. As a result of this a depletion of spermatocytes was observed 14 days after injection of busulfan, while after this interval Leydig cells appeared normal (Fig. 3b). These findings confirm the results of an earlier study in which it was shown that this dose of busulfan kills all types of A spermatogonia (de Rooij and Kramer, 1970). This results in a depletion of the In and part of the B spermatogonia after 3 days and part of the spermatocytes after 14 days.

Effects of EDS on Leydig cells were also studied in immature (22-day-old) rats and mature mice 72 h after administration of EDS. In frozen sections from immature rats, the number of 3β-HSD-positive cells present in the interstitium of the testis from control and EDS-treated animals was not different. Phenyl esterase activity, expressed as phenol production per testis, was 8.8 ± 1.5 and 11.2 ± 1.5 nmol·min^{-1}·testis^{-1} for control and EDS-treated mice, respectively (means ± SD, n=4).

The basal and LH-stimulated steroid production by interstitial cells isolated from immature

TABLE 1. Testicular phenyl esterase activity at various times after administration of EDS (75 mg/kg body weight).

Treatment	Hours after treatment	Tissue	Phenol production (nmol·min^{-1}·mg protein^{-1})[a]
None	–	Total testis	119.8 ± 10.3 (4)
Vehicle	72	Total testis	110.8 ± 7.9 (3)
EDS	16	Total testis	71.6 ± 8.2 (3)*
EDS	48	Total testis	12.2 ± 4.3 (3)*
EDS	72	Total testis	8.2 ± 0.5 (3)*
hCG + EDS[b]	72	Total testis	24.0 ± 21.4 (9)*
None	–	Dissected tubules	19.5 ± 0.2 (3)*

[a]Means ± SD. Number of rats in parentheses.

[b]3 days' pretreatment with hCG (100 IU); at Day 4 hCG + EDS.

*Significantly different from control (P<0.005).

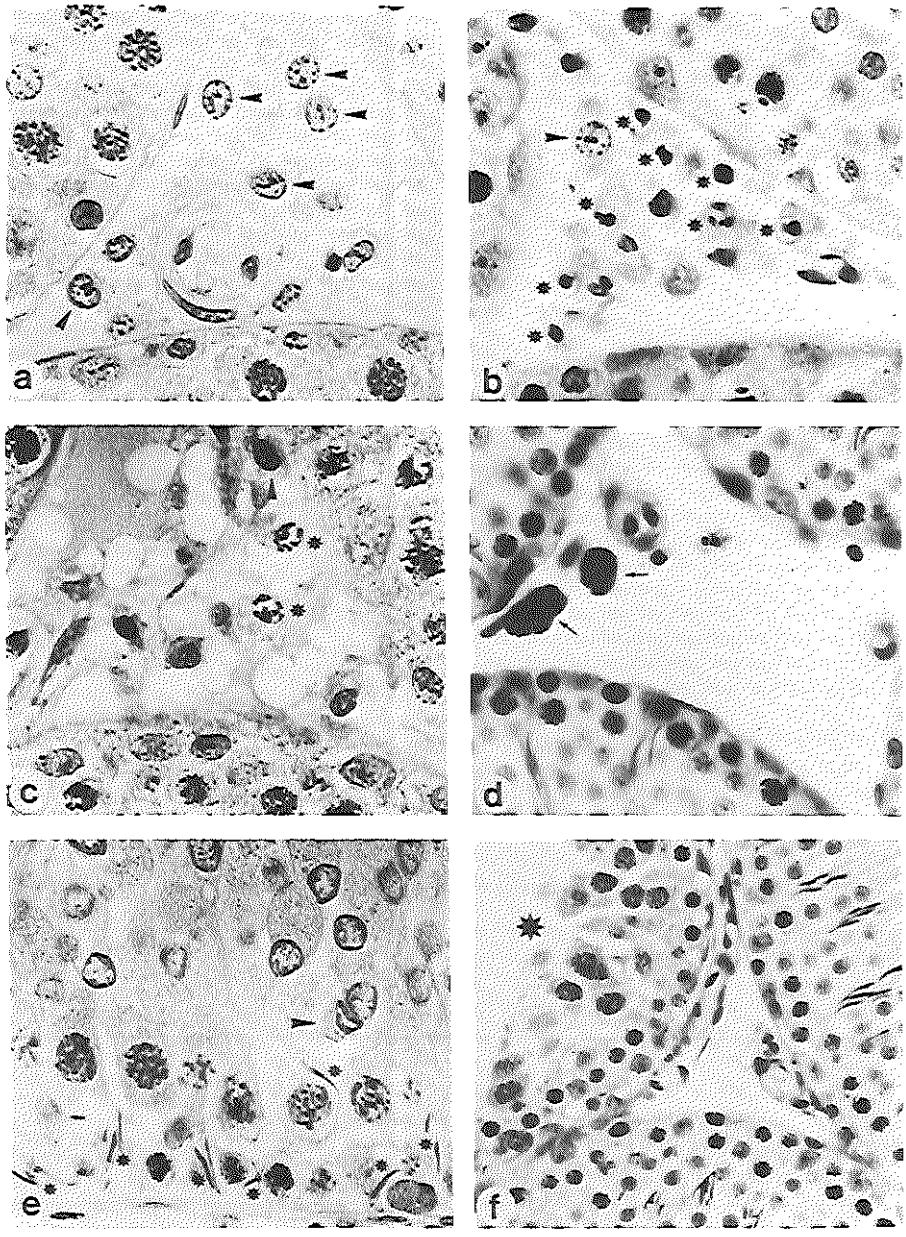
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FIG. 1. (*a*) Interstitium of a rat 3 days after injection of DMSO. No effect on Leydig cells (*arrowheads*) was observed. ×720. (*b*) Interstitium of a rat 16 h after injection of EDS. Numerous degenerating Leydig cells (*asterisks*), showing pycnotic nuclei, were observed. Some Leydig cells still looked normal (*arrowhead*). ×720. (*c*) Interstitium 48 h after injection of EDS. Most interstitial spaces did not show Leydig cells, but, in a few, degenerating cells (*asterisks*) and occasionally a normal Leydig cell (*arrowhead*) were observed. ×720. (*d*) Interstitium 72 h after injection of EDS. After this interval Leydig cells were virtually absent. Macrophages (*arrows*), in this case labeled with trypan blue, were still numerous. ×600. (*e*) Interstitium 7 days after injection of EDS. After this interval an effect of the treatment on the seminiferous epithelium was observed. Many degenerating step 19 spermatids, lying close to the basement membrane, were observed (*asterisks*). In this tubular section the epithelium is in early stage IX, so these spermatids should already have been released into the tubular lumen. Degenerating elongating spermatids were also seen. One of these, having a grossly abnormally shaped nucleus and undergoing karyolysis, is shown (*arrowhead*). ×720. (*f*) Interstitium 7 days after injection of EDS. One of the tubular sections (*asterisk*) shown here is in epithelial stage X–XIII. Note the absence of elongating spermatids, making it impossible to determine the exact epithelial stage. ×375.

rats and mice was not inhibited by the EDS treatment (Table 3).

Protection by hCG

In rats treated with hCG for 3 days before injection of EDS, Leydig cells were present in the interstitium 72 h after administration of EDS. This is clearly different from the situation 72 h after treatment with EDS alone, when all Leydig cells had disappeared. In 3 animals numerous Leydig cells were seen in the interstitium (Fig. 3c), whereas in 2 animals the number of Leydig cells seemed lower.

Plasma testosterone concentrations were increased approximately 10-fold after 3 days'

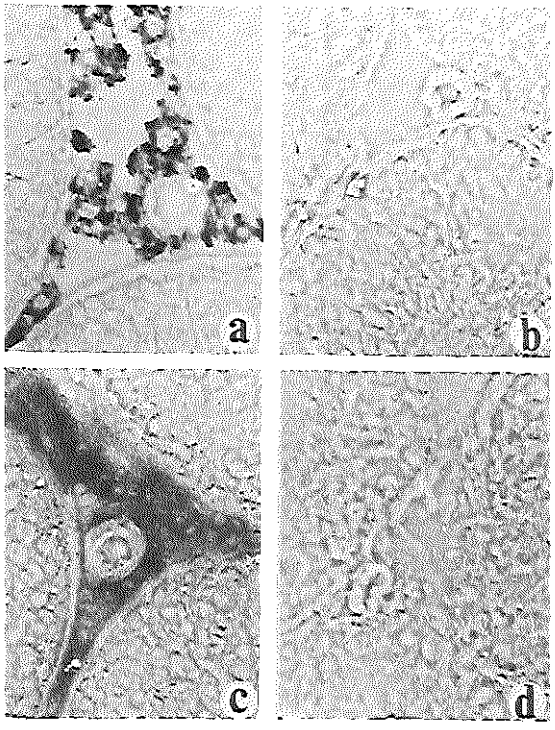

FIG. 2. (*a,b*) Testis of a rat 3 days after injection of DMSO (*a*) or EDS (*b*) stained for α-naphthyl esterase activity. ×250. (*c,d*) Testis of a rat 3 days after injection of DMSO (*c*) or EDS (*d*) stained for 3β-hydroxysteroid dehydrogenase. ×250.

TABLE 2. Plasma testosterone concentrations at various times after administration of EDS (75 mg/kg body weight).

Treatment	Hours after treatment	Testosterone (nmol/l)[a]
None	–	6.7 ± 4.2 (12)
Vehicle	72	7.6 ± 3.5 (4)
EDS	16	1.9 ± 0.7 (11)*
EDS	48	0.1 ± 0.1 (6)*
EDS	72	0.4 ± 0.3 (8)*
hCG	72	78.7 ± 27.0 (12)*
hCG + EDS[b]	72	10.5 ± 12.8 (9)**

[a]Means ± SD. Number of rats in parentheses.

[b]3 days' pretreatment with hCG (100 IU); at Day 4 hCG + EDS.

*Significantly different from control ($P<0.005$).

**Significantly different from hCG-treated control ($P<0.005$).

daily injection with hCG. Administration of hCG + EDS on Day 4 resulted in an approximately 8-fold decrease in testosterone concentrations 72 h later, but these values were not different from those in untreated controls (Table 2). However, the specific activity of esterase was decreased to approximately 20% of the control value (Table 1). Large differences in protection by hCG were observed between different animals, but a relationship between the decrease in plasma testosterone, esterase activity, and the number of Leydig cells present in sections was observed.

DISCUSSION

Our results obtained with ethane dimethyl sulfonate (EDS) demonstrate that this antifertility agent, in contrast to other members of the group of methyl sulfonic acid esters, acts via the Leydig cells. The damaging effects of EDS on the Leydig cells in testes from mature rats occur very rapidly. Plasma testosterone levels were already significantly decreased 16 h after EDS treatment, which reflects a rapid loss in functional activity of the Leydig cells. At that time Leydig cells with pycnotic nuclei were seen in the interstitium, indicating degeneration of these cells. Phenyl esterase activity, a marker for Leydig cells (Niemi et al., 1966; Meyer et al., 1974; Cooke et al., 1981; Molenaar et al., 1983), was measured in testis tissue for a quantitative evaluation of the destructive effect of EDS. The specific activity of the testicular esterase also was decreased 16 h after administration of EDS.

A further decrease in the plasma testosterone concentration was observed between 16 and 48 h after EDS, and the concentration remained low until 72 h. This is in agreement with observations from Morris and McCluckie (1979), who found a drop in androgen levels almost to the level of castrated adrenalectomized rats within 24 h. At 48 h after treatment, the esterase activity was even lower than the activity present in dissected tubules of normal rats. This indicates a total loss of Leydig cell activity. The higher activity of the

TABLE 3. Steroid production by interstitial cells isolated from rats and mice (pregnenolone in rats and testosterone in mice) 72 h after EDS treatment (75 mg/kg body weight).

Interstitial cell source	Treatment	Steroid production (pmol·10^6 cells^{-1}·h^{-1})[a] Basal	LH (100 ng/ml)
Mature rats	Vehicle	4.2 ± 2.2 (3)	44.7 ± 16.5 (3)
	EDS	0.4 ± 0.2 (3)	0.3 ± 0.2 (3)*
Immature rats	Vehicle	8.0 ± 1.6 (3)	129.6 ± 1.6 (3)
	EDS	8.8 ± 0.8 (3)	142.4 ± 8.0 (3)
Mature mice	Vehicle	70.4 ± 39.2 (3)	1618.3 ± 920.2 (3)
	EDS	141.2 ± 127.4 (3)	1302.6 ± 1023.2 (3)

[a]Means ± SD. Number of cell preparations in parentheses.

*Significantly different from control ($P<0.005$).

TABLE 4. Phenyl esterase activity in testis tissue from mature rats 72 h after treatment with various sulfonic esters.

Compound	Phenol production (nmol·min^{-1}·mg protein^{-1})[a]
Vehicle	87.2 ± 5.9 (2)
EMS	89.6 ± 0.2 (2)
Busulfan	68.5 ± 2.6 (2)
EDS	8.1 ± 0.1 (2)

[a]Means ± range; number of rats in parentheses.

dissected tubules from normal rats can be explained by a small contamination with Leydig cells. At 48 h after EDS only a few normal Leydig cells were left in the interstitium and after 72 h virtually no Leydig cells could be detected. The difference in the start of degeneration between individual cells may indicate that Leydig cells differ in their sensitivity to EDS.

It has been demonstrated by several authors that hypophysectomy (Hsueh et al., 1976; Purvis et al., 1978) or suppression of LH (action) (Dym and Madhwa Raj, 1977; Gondos et al., 1980; Wing et al., 1984) causes a loss of Leydig cell activity as reflected by a decrease in steroid production and morphologic changes, but Leydig cells remain present in the interstitium. The effects of low LH levels are thus clearly different from the effects of EDS, which results in the destruction of Leydig cells. Furthermore, Jackson and Morris (1977) have demonstrated that the serum LH concentration increases following EDS treatment. Moreover, addition of EDS to isolated pituitary cells did not affect the LH and FSH secretion (de Jong et al., unpublished results), whereas EDS added to Leydig cells inhibits LH-stimulated steroid production within 3 h (Rommerts et al., 1985).

Hence, we have concluded that the effects of EDS are not mediated via the pituitary, but that EDS acts directly on the Leydig cells.

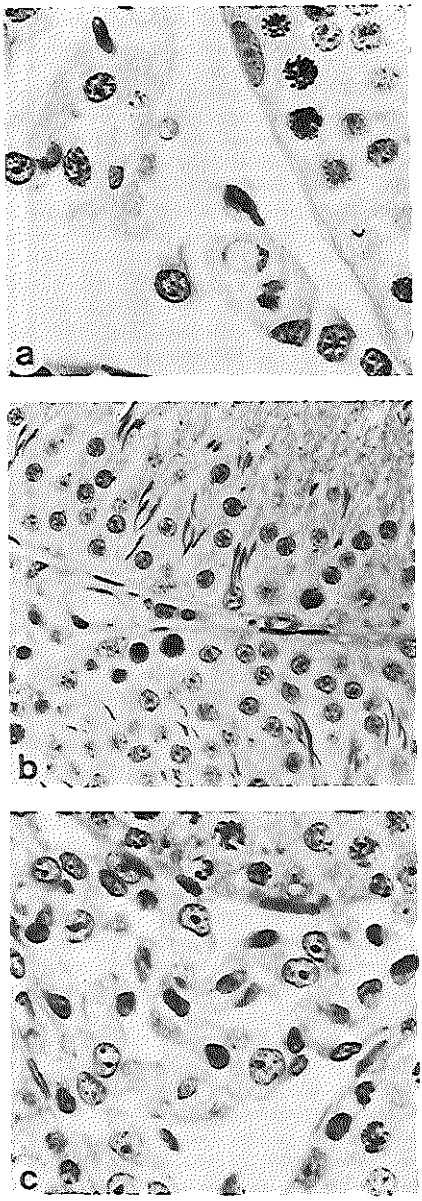

FIG. 3. (*a*) Interstitium of a rat 3 days after injection of busulfan. No effect on Leydig cells was observed. ×720. (*b*) Seminiferous tubules 14 days after injection of busulfan. Busulfan does have an effect on spermatogonia, which can be deduced from the sparsity of pachytene spermatocytes in both tubules. ×400. (*c*) Interstitium of a rat 3 days after injection of EDS; the rat was pretreated with hCG for 3 days before injection with EDS. Not all rats responded similarly, but in this one numerous Leydig cells were present at this interval after injection of EDS. ×600.

Moreover, a clear difference in sensitivity to EDS was observed between Leydig cells from mature rats and those from immature rats and mice. Interstitial cells isolated from mature rats 72 h after EDS treatment could not be stimulated by LH. Also, the yield of interstitial cells from EDS-treated rats was much lower than from control rats and 3β-HDS- and α-NE-positive cells were not present either in the isolated cell preparation or in frozen testis sections. In contrast, no effect of EDS treatment was observed on 3β-HSD and α-NE activity and steroid production by interstitial cells isolated from immature rats and mature mice. The macrophages in the interstitium also appeared to be resistant to EDS. These data suggest that EDS acts selectively on Leydig cells from mature rats. Jackson and Jackson (1984) have found that Leydig cells from mice are resistant to EDS, while Jones et al. (1972) have demonstrated that EDS does act on Leydig cells from the Japanese quail. These data indicate that the action of EDS depends on the species and the stage of maturation of the animals. Rommerts et al. (1985) have demonstrated that the presence of EDS in vitro also inhibits steroid production by Leydig cells isolated from mature rats without affecting steroid production by Leydig cells from immature rats and mice. Furthermore, the specificity of EDS was demonstrated since busulfan and EMS, compounds with similar structures, had no effects on Leydig cell morphology and phenyl esterase activity of testis tissue.

One week after treatment with EDS spermatogenesis was affected. Spermiation appeared to be disturbed, as many degenerating step 19 spermatids were seen in stages VII–XII. Furthermore, degenerating step 9–11 spermatids were seen in the corresponding stages as well as some degenerating pachytene spermatocytes in stages VII and VIII. This effect closely resembles that described by Clermont and Morgentaler (1955) and Russell and Clermont (1977) 1 wk after hypophysectomy. Hence, testosterone deprivation due to the disappearance of Leydig cells may well be the cause of the degeneration of these germ cells.

The present results show that pretreatment with hCG can protect the Leydig cells morphologically. At 72 h after treatment with hCG + EDS, testosterone concentrations in plasma were similar to the control values. Since the biologic half-life of testosterone is approximately 10 min (de Jong et al., 1973), this indicates that testosterone is still produced by functionally active Leydig cells. In contrast, a decrease in esterase activity was observed. It has been demonstrated that treatment with hCG results in an increase in the number of Leydig cells within 1 wk (Chemes et al., 1976; Christensen and Peacock, 1980). Pretreatment with hCG in our experiments might have led to an increase in the number of Leydig cells with "immature" characteristics, which are resistant to EDS and are low in esterase (Niemi and Ikonen, 1963; Meyer et al., 1974), although this was not apparent in the morphology. Moreover, the protective action of hCG was variable, as reflected in the large differences observed between individual animals. Hence, the mechanism responsible for the partial protective action of hCG is still unknown and further studies are required to elucidate the basis for the protection by hCG.

In this study we have demonstrated that EDS can destroy specifically Leydig cells from mature rats. Several studies (Jackson and Morris, 1977; Morris and McCluckie, 1979; Jackson and Jackson, 1984) have reported that the effect of EDS on the testis and accessory organs is temporary. This suggests that either a few Leydig cells or precursor cells remain in the testis after administration of EDS. Since no 3β-HSD-positive cells were present 72 h after EDS treatment, we have concluded that all Leydig cells were killed and that the precursor cell did survive. Many authors have speculated about the nature of this precursor cell; it has been thought to be a mesenchymal cell (Moon and Hardy, 1973; Gondos et al., 1975), a fibroblast-like cell (Chemes et al., 1976), and a macrophage (Clegg and McMillan, 1965; Christensen, 1975). Ethane dimethyl sulfonate might be of great help in studies concerning the origin and regulation of development of Leydig cells.

Moreover, EDS offers the possibility to create a "Leydig cell-depleted" testis, which, comparable to the Sertoli cell-enriched testis (Beaumont, 1960; Rich and de Kretser, 1977; Slaughter and Means, 1983), may offer a very useful model for further studies concerning the role of Leydig cells in local regulation of testis functions. Such studies could concern the increase in permeability of the testicular capillaries caused by hCG (Setchell and Sharpe, 1981; Sharpe, 1983), which is supposed to be mediated via the Leydig cell, and the immunosuppressive activity of the testis (Head et al., 1983), which is suggested to be caused by the

production of immunosuppressive compounds by the Leydig cells (Born and Wekerle, 1982; Staples et al., 1983).

ACKNOWLEDGMENTS

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APPENDIX PAPER IV

Endocrinology, in press

REPOPULATION OF LEYDIG CELLS IN MATURE RATS AFTER SELECTIVE DESTRUCTION OF THE EXISTENT LEYDIG CELLS WITH ETHYLENE DIMETHANE SULFONATE (EDS) IS DEPENDENT ON LH AND NOT FSH

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ABSTRACT

After selective destruction of Leydig cells in mature rats with Ethylene Dimethane Sulfonate (EDS) repopulation of Leydig cells occurs. This repopulation process was studied in normal and sterile (prenatally irradiated) rats using morphological and histochemical techniques and by measuring hormone concentrations. 3 Days after administration of EDS to normal rats extensive Leydig cell degeneration had occurred, testosterone concentrations were decreased to less than 10% of the normal value and no 3 β-Hydroxy Steroid Dehydrogenase (3β-HSD) activity or pregnenolone production could be detected in isolated interstitial cells. 7 Days after EDS administration no cells with the appearance of Leydig cells were observed and steroidogenic activities were still absent. After 14 days single or paired Leydig cells were present again in the interstitium, but only after 21 days an increase in the plasma testosterone concentration and LH dependent pregnenolone production was observed. At day 35 numerous Leydig cells were present and testosterone levels were restored to normal.

The depletion and repopulation of Leydig cells after administration of EDS to sterile rats showed a somewhat different pattern. 3 Days after administration of EDS testosterone concentrations were decreased to less than 10% of the normal value and isolated interstitial cells showed no steroidogenic activities like in normal rats, but a small number of Leydig cells was still present. A similar picture was observed between 4 and 9 days after EDS administration. This indicates that some Leydig cells from sterile rats, unlike Leydig cells from normal rats, were resistant to EDS. The repopulation of Leydig cells in sterile rats was faster than in normal rats. After 14 days many groups of Leydig cells were present in the interstitium and the plasma testosterone concentration and pregnenolone production in vitro were significantly increased. Normal plasma testosterone levels were restored at day 21.

Serum LH and FSH were decreased immediately after EDS administration, but during the next days a sharp rise was observed in both normal and sterile rats. The rise in LH correlated with the decrease in testosterone and restoration of LH levels took place when testosterone levels increased. FSH levels changed similarly, but delayed, in comparison to LH.

In rats with testosterone implants which suppressed LH levels to less than 2ng/ml and maintained normal FSH levels, ranging from 150 to 340 ng/ml, as well as in hypophysectomized rats, no repopulation of Leydig cells could be observed until 35 days following EDS treatment. Daily injections with hCG to these rats, beginning at day 3 after EDS administration, stimulated the repopulation and Leydig cells with apparently normal morphology and steroidogenic activities were present at day 35. This implies that FSH, prolactin and other pituitary hormones are not required for repopulation. Treatment of hypophysectomized rats with FSH alone was uneffective.

These results demonstrate that there are precursor cells for Leydig cells in the testis which are resistant to EDS and are dependent on LH for their multiplication and differentiation to proper Leydig cells. In contrast to Leydig cell differentiation during normal sexual maturation, FSH is not required for development of Leydig cells in mature rats after treatment with EDS.

INTRODUCTION

Recently it has been demonstrated that testis Leydig cells in mature rats can be selectively destroyed by treatment with Ethylene Dimethane Sulfonate (EDS) (1,2,3). Studies from Jackson and Morris (4) and Morris and McCluckie (5) have shown that the effect of EDS on testosterone production and secondary sex organs is temporary. Since EDS treatment causes an impressive destruction of Leydig cells these latter results strongly indicate that restoration of testosterone production

and secondary sex characteristics reflect a repopulation of Leydig cells.

It is generally accepted that normal differentiation of Leydig cells requires the presence of both LH (6,7) and FSH (8,9,10,11,12). Since FSH acts on Sertoli cells (13,14) this suggests that, in addition to LH, local factors probably produced by Sertoli cells are involved in the development of Leydig cells.

In the present study we have investigated the repopulation of Leydig cells, following EDS treatment in normal mature rats and in sterile (germ cell depleted) mature rats, where paracrine regulation may be different because of an altered Sertoli cell function (15). Furthermore the possible role of gonadotrophic hormones in this repopulation was studied.

MATERIALS AND METHODS

Materials

Ethylene Dimethane Sulfonate (EDS), which is not commercially availabe, was synthesized as described by Jackson and Jackson (16) from ethylene glycol and methane sulphylchloride. Human chorionic gonadotropin (hCG) (Pregnyl) was obtained from Organon, Oss. Ovine FSH (NIAMDD-FSH-S13) and ovine LH (NIH-LH-S18, 1.03 i.u./mg) were a gift from the National Institute of Health, Bethesda, Md., U.S.A.

Treatment of animals

3 to 5 month-old male Wistar rats and 4 to 12 month-old sterile rats, obtained by irradiation at day 20 of gestation with a dose of 1.5 Gy of X-rays (17) were used. EDS (30 mg/ml in DMSO:H_2O, 1:3) was administered in one intraperitoneal injection (75 mg/kg body weight). As a control a single intraperitoneal injection of the vehicle was given. In some rats in addition to EDS, a silicone elastomere capsule (length: 5 cm; inside $\oslash$ 1.5 mm; outside $\oslash$ 2.1 mm) (Rubber,

Hilversum, The Netherlands) filled with testosterone was implanted subcutaneously on the back. Other rats were hypophysectomized and received the EDS the next day. The effect of the hypophysectomy was checked at the end of the experiment by measuring plasma corticosterone. Plasma corticosterone levels were always less than 20 nmol/l. HCG and FSH treatment was started 3 days following EDS treatment. Rats were injected daily s.c. with hCG (15 i.u.) (dissolved in 0.9% NaCl) or FSH (5 µg) (dissolved in 50% polyvinyl pyrrolidon) or the vehicle (control).

Measurements

Blood was collected either by orbital punction under ether anesthesia for estimation of LH, FSH and testosterone, or after decapitation for estimation of testosterone and corticosterone. Plasma was stored at -20°C. Testes were removed immediately after killing the animals via decapitation. For histology testis tissue was fixed in Bouin's fluid and 5 µm paraffin and butoxy ethanol-glycol methacrylate sections were made and stained by the PAS technique and hematoxylin. Testosterone was measured in plasma by radioimmunoassay according to Verjans et al. (18). LH and FSH were measured in plasma as described by Welschen et al. (19).

For preparation of interstitial cells testes were decapsulated and treated with collagenase as described by Rommerts et al. (20). Pregnenolone production was measured in the incubation media in the presence of inhibitors of pregnenolone metabolism according to Rommerts et al. (21). Measurements of pregnenolone are as close as possible to the rate limiting step of steroidogenesis, the cholesterol side chain cleavage, and are independent of pregnenolone converting enzymes. 3β-Hydroxy Steroid Dehydrogenase (3β-HSD) histochemistry was performed according to Molenaar et al. (22).

For statistical analysis Students t-test for paired data was used in LH and FSH measurements. For other parameters

Students t-test for unpaired data was used. Differences were considered to be statistically significant when $p<0.01$.

RESULTS

Repopulation of Leydig cells in normal and sterile mature rats

Morphology

In the normal rats 3 days after administration of EDS sporadically a Leydig cell was observed (fig. 1a), and after 7 days no cells with the appearance of Leydig cells were seen. However 14 days after administration of EDS single or paired Leydig cells were present again (fig. 1d). Occasionally a group of 3 or 4 Leydig cells was seen. After an interval of 21 days larger groups of more than 10 Leydig cells were observed and after 35 days the interstitium contained numerous Leydig cells comparable to control testes (fig. 1f).

In the sterile rats a somewhat different pattern of depletion and repopulation was seen after administration of EDS. Although after 3 days the number of Leydig cells was greatly reduced, some Leydig cells were still present in the interstitial spaces (fig. 1b) and also after 7 days, unlike in normal rats, occasionally cells with the appearence of Leydig cells could be found (fig. 1c). After 14 days many new Leydig cells lying in groups of sometimes more than 10 cells were observed again (fig. 1e). Like in normal rats numerous Leydig cells were present after 35 days (fig. 1g).

In order to investigate whether the Leydig cells seen in testes of sterile rats 7 days after administration of EDS represented newly formed Leydig cells or cells that survived the treatment with EDS, an additional experiment was done in which testes were collected at day 4,5,6,8,9 and 11 following administration of EDS. Occasionally cells with the appearance of Leydig cells were seen between 4 to 9 days, like the picture at day 7. After 11 days the first signs of repopulation were apparent as more single and also small

groups of 2-4 Leydig cells were observed.

Plasma testosterone, LH and FSH concentration

Plasma testosterone concentrations, reflecting the presence of active Leydig cells, were measured at different intervals after EDS treatment (fig. 2a,b). In both normal and sterile rats testosterone levels were decreased to approx. 10% of the original value 3 days after administration of EDS. In normal rats levels remained low up to day 14 and control levels were reached from day 22 onwards. In sterile rats a more rapid recovery following the treatment with EDS was observed. A slight increase in the plasma testosterone concentration was already observed at day 7 and at day 14 a significant ($p<0.025$) elevation was observed compared to day 3. At day 22 normal testosterone concentrations were reached. After administration of EDS to normal rats LH levels rose, after an initial dip at day 1 and reached a maximum at day 14 (approx. 900% of the original value) (fig. 2c). In sterile rats the maximum was reached at day 7 (approx. 400% of the original value) (fig. 2d). The absolute concentrations measured at day 0, 3 and 7 were significantly higher ($p<0.005$, $p<0.05$ and $p<0.005$ respectively) in sterile rats when compared to normal rats. Normal LH levels were reached again at day 22 in both normal and sterile rats at the same time as the restoration of plasma testosterone took place.

The effect of EDS on plasma FSH levels were essentially similar to those on LH, but somewhat delayed. Maximal levels were reached in normal rats at day 22 (approx. 300% of the original value) (fig. 2e) and in sterile rats at day 14 (approx. 200% of the original value) (fig. 2f). In normal rats the concentration was still elevated at day 35, whereas in sterile rats the normal concentration was reached at day 22. The absolute concentrations of FSH in sterile rats were also significantly higher at day 0, 3, 7 and 14 ($p<0.010$) than in normal rats.

No significant effects of the vehicle DMSO on plasma

a
b
c
d
e
f
g

Figure 1

a. Interstitium of a normal rat 3 days after injection of EDS. No Leydig cells were observed. 330x

b. Interstitium of a sterile rat 3 days after injection of EDS. Although their number is greatly reduced, some Leydig cells can still be seen (arrow heads). 680x.

c. Interstitium of a sterile rat 7 days after injection of EDS. Leydig cells are rare after this interval. Occasionally a cell with the appearance of a Leydig cell can be found (arrow head). 900x.

d. Interstitium of a normal rat 14 days after injection of EDS. Leydig cells lying isolated or in pairs can be seen, rarely a group of 3 or 4 cells is observed (arrow head). 640x.

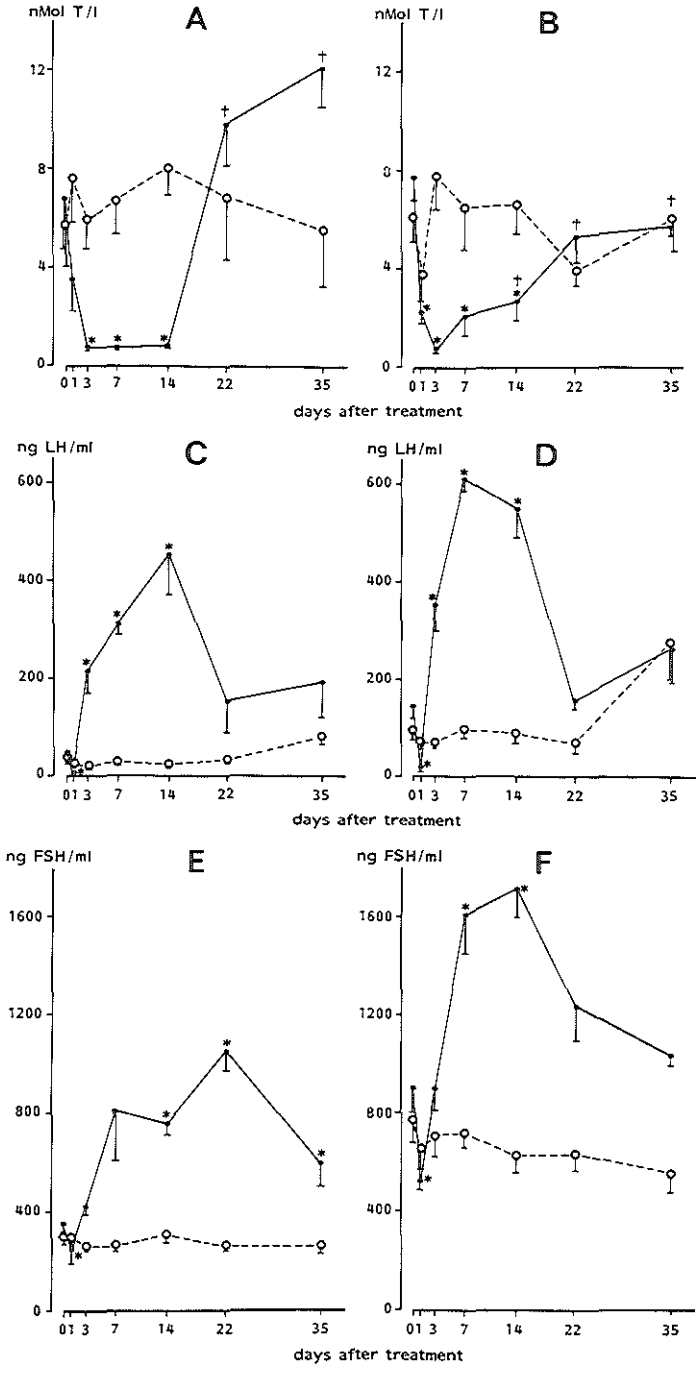
e. Interstitium of a sterile rat 14 days after injection of EDS. Clusters of sometimes more than 10 Leydig cells are present. Three cells of such a cluster are indicated (arrow heads). 640x.

f,g Interstitium of a normal (f) and sterile (g) rat 35 days after injection of EDS. Numerous Leydig cells are present in the interstitium. f-370x; g-330x.

Bar - 10 µm.

Figure 2

Temporal changes in concentrations of serum testosterone (T) (A,B), LH (C,D) and FSH (E,F) concentrations in normal (left) and sterile (right) rats following administration of EDS (•——•) or vehicle (o--o). Means ± S.E.M., n>5
*significantly different from day 0


nMol T/l
A
B
12
8
4
0
0 1 3 7 14 22 35
days after treatment
ng LH/ml
C
D
600
400
200
ng FSH/ml
E
F
1600
1200
800

testosterone, LH and FSH concentrations were observed.

Activities of isolated cells

The steroidogenic capacity of interstitial cells isolated at different intervals after EDS treatment was studied by measuring the LH stimulated pregnenolone production in vitro (fig. 3). No pregnenolone production could be detected in preparations isolated from normal rats from day 3 to day 14. LH-stimulated production was detectable in cell preparations isolated at day 21 and a normal production was obtained at day 35. In preparations isolated from sterile rats no LH-stimulated production was detected 3 days after administration of EDS. Cell preparations obtained at 7 days after EDS indicated a transition phase. In some preparations containing 3β-HSD positive cells significant LH-dependent steroid production was observed, whereas in other cell preparations this could not be shown. Due to this variation the mean pregnenolone production was not significantly elevated at this time point. In preparations isolated at 14 days after EDS, the LH-stimulated pregnenolone production was significantly elevated and at day 21 a normal production was obtained. This was correlated with an increase in the number of 3β-HSD positive cells (fig. 4). 35 Days following EDS the percentage 3β-HSD positive cells was not significantly different from the percentage present in control preparations from normal and sterile rats.

These data on steroidogenic activities of cell preparations isolated after EDS confirm the morphological data and show that restoration of functional properties of Leydig cells in sterile rats occur more rapid than in normal rats.

Effect of gonadotrophins on repopulation of Leydig cells

The role of LH and FSH in the repopulation of Leydig cells was examined. Rats with a subcutaneously implanted testosterone filled capsule showed strongly suppressed LH levels (less than 2 ng/ml) and normal FSH levels (ranging from 150 to 340

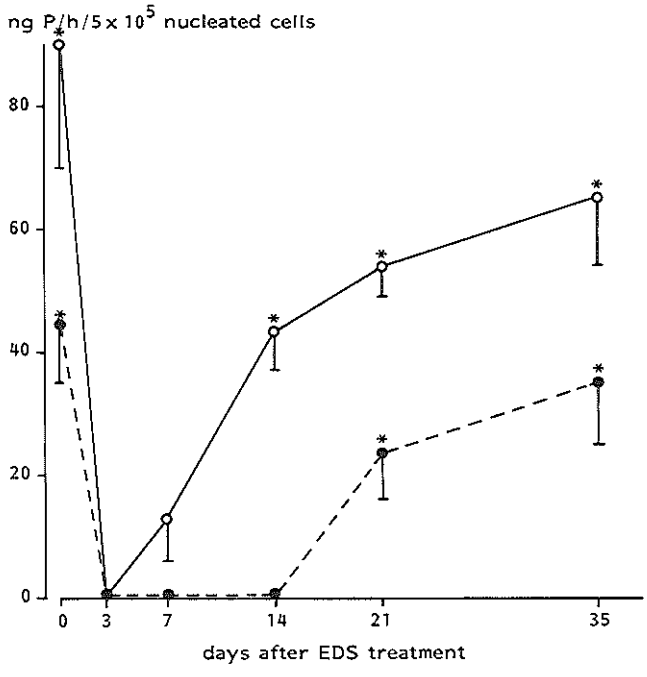


Figure 3
LH-stimulated pregnenolone (P) production by cell preparations isolated from normal (●- - -●) and sterile (○——○) rats following EDS treatment. Means ± S.E.M., n=3-6.
*significantly different from day 3.
Normal values were restored from day 21 onwards. No effect of the vehicle alone was observed: the production by preparations from normal rats (2, 3 and 7 days following administration) was 31.0 ± 2.2 (Means ± S.E.M., n=3).

ng/ml). In these rats there was no evidence for Leydig cell repopulation 35 days after EDS. This was based on morphological observations (fig. 5a), and pregnenolone production and histochemical 3β-HSD activity of isolated cells (Table 1). After daily hCG treatment, many Leydig cells were observed in histological sections at day 35 (fig. 5b) and many 3β-HSD positive cells were present in the isolated cell preparation (data not shown). Furthermore, the pregnenolone production in vitro by cells isolated at day 35 increased

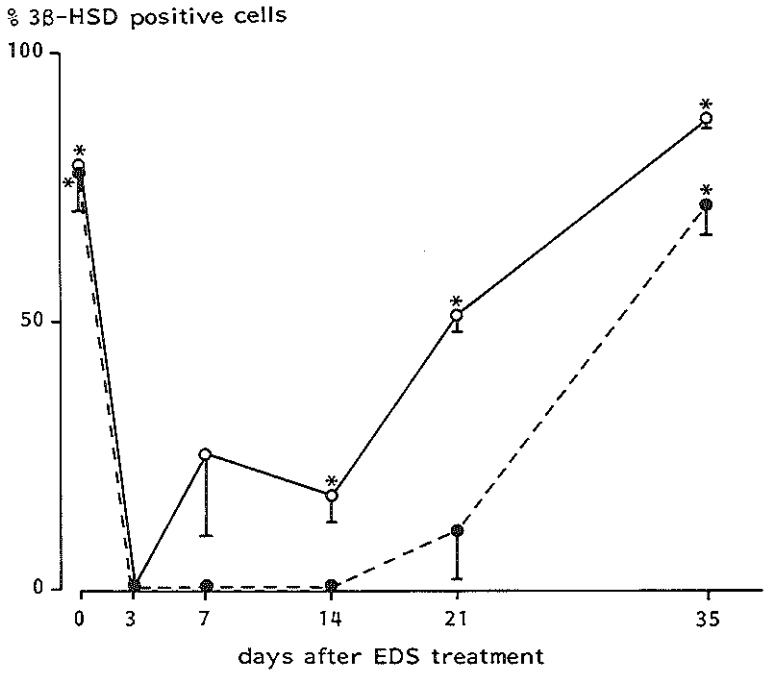


Figure 4
Percentage 3β-Hydroxy Steroid Dehydrogenase (3β-HSD) positive cells in cell preparations isolated from normal (●- - -●) and sterile (o——o) rats following EDS treatment.
Means ± S.E.M., n=3-6.
*significantly different from day 3.
Normal values were restored on day 35.

after addition of LH and 22R-OH-cholesterol approx. 2-fold and 10-fold respectively (Table 1). The stimulation factor was rather low which might be caused by elevation of the basal production as the result of the hCG treatment. 21 Days after EDS and hCG treatment pregnenolone producing cells could already be isolated (Table 1), but the production by cells isolated 35 days following EDS was higher than by cells isolated at day 21.

The possible effects of hCG and of FSH alone on Leydig cell development were investigated in hypophysectomized rats

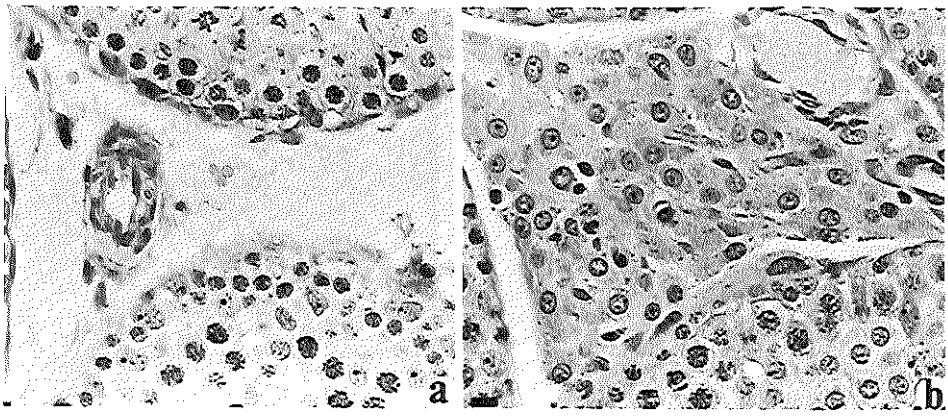


Figure 5
Interstitium of rats with suppressed endogenous LH, 35 days following injection of EDS. 420x; Bar - 10 µm.
a. Interstitium of rats that received no further treatment. No Leydig cells are present.
b. Interstitium of rats receiving hCG. Very large clusters of Leydig cells are present.

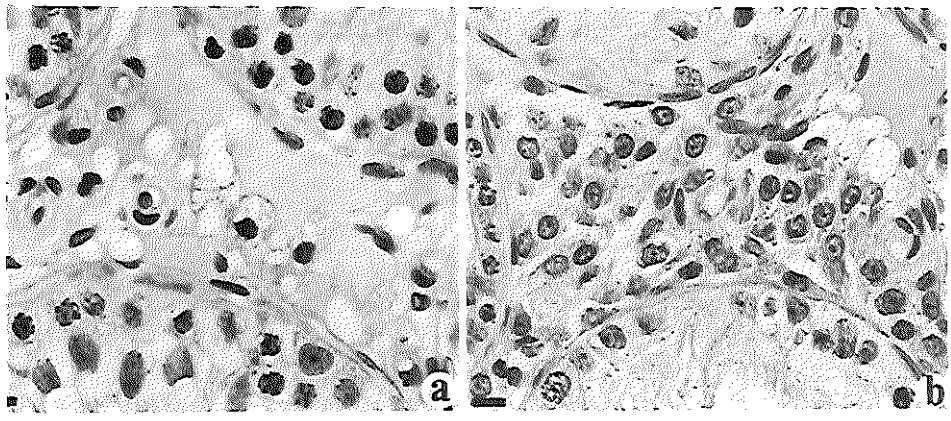


Figure 6
Interstitium of hypophysectomized rats 35 days following injection of EDS. Bar - 10 µm.
a. Interstitium of rats receiving FSH. No Leydig cells can be discerned. 650 x.
b. Interstitium of rats receiving hCG. Numerous Leydig cells are present. 530x.

Table 1: Effect of hCG treatment on pregnenolone (P) production by interstitial cell preparations isolated from rats with suppressed endogenous LH, following EDS treatment

treatment	days after EDS	pMol P/3x10^5 cells/h		
		basal	LH stimulated (100 ng/ml)	in the presence of 22R-OH-cholesterol
control	21	<0.8 (8)	<0.8 (4)	2.4 ± 0.1 (4)
hCG	21	5.4 ± 0.6 (8)*	12.5 ± 1.3 (4)*	77.0 ± 17.3(4)*
control	35	<0.8 (8)	<0.8 (4)	1.1 ± 0.1 (4)
hCG	35	17.2 ± 4.8 (8)*	50.2 ± 25.8 (4)*	196.3 ± 48.1 (4)*

Means ± S.D. Number of incubations of 2 different cell preparations in parentheses.
* significantly different from control.

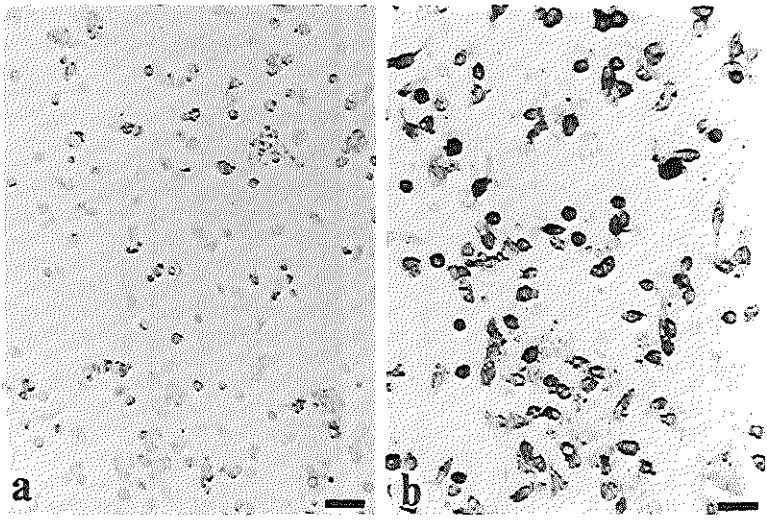


Figure 7
Interstitial cell preparations isolated from hypophysectomized rats 35 days following EDS treatment, stained for 3β-Hydroxy Steroid Dehydrogenase (3β-HSD) activity. 500x; Bar - 10 µm.
a. FSH treated rats. No 3β-HSD positive cells were observed.
b. HCG treated rats. Numerous 3β-HSD positive cells were observed.

being completely devoid of gonadotrophic hormones. Similar to the results obtained with untreated rats with only suppressed LH levels, repopulation of Leydig cells did not occur in untreated hypophysectomized rats and in hypophysectomized rats treated with FSH (fig. 6, 7, Table 2). Daily hCG treatment following EDS resulted in a clear repopulation of Leydig cells at day 35. Large numbers of Leydig cells were present in the interstitium (fig. 6b) and plasma testosterone concentrations were significantly increased (Table 2). Many 3β-HSD positive cells were present in isolated cell preparations (fig. 7b) and the pregnenolone production by these cell preparations was stimulated by LH and 22R-OH-cholesterol approx. 3- and 14-fold respectively (Table 2). These data show that repopulation of Leydig cells requires LH and not FSH.

Table 2: Effect of hCG and FSH treatment of hypophysectomized rats on steroidogenic activities 35 days following EDS

treatment	plasma testosterone (nmol/l)	pregnenolone production in vitro pMol/3x10^5 nucleated cells/h		
		basal	LH	22R-OH-cholesterol
vehicle	<0.9 (7)	0.9 ± 0.4 (6)	6.9 ± 9.0 (6)	36.3 ± 40.4 (5)
hCG	36.4 ± 24.6* (3)	104.3 ± 57.5* (3)	308.8 ± 171.4* (3)	1491.9 ± 83.2* (3)
FSH	<0.9 (3)	0.6 ± 0.1 (3)	0.8 ± 0.1 (3)	4.1 ± 1.2 (3)

Means ± S.D. Number of cell preparations in parentheses.
* significantly different from control ($p<0.005$)

DISCUSSION

After treatment of normal mature rats with Ethylene Dimethane Sulfonate (EDS) no Leydig cells were detected in the testis based on morphological criteria, confirming our earlier results (3). A new population of Leydig cells was developing between day 7 and 14 after administration of EDS, which suggests the presence of a population of EDS resistant precursor cells which cannot be identified as Leydig cells, at least by the morphological and histochemical methods used in this study. The nature of this precursor cell has not been elucidated yet, but several authors have suggested that this might be a cell of mesenchymal origin (11,12,23,24,25), which is also named: fibroblastlike cell (26,27) fibroblastlike-endothelial cell (28) and indeterminate connective tissue cell (29).

Selective suppression of endogenous LH, by testosterone filled implants, prevented the repopulation of Leydig cells up to 35 days after EDS treatment as was seen by the absence of Leydig cells in testis sections and the lack of steroidogenic activities in isolated cell preparations. Daily treatment with hCG clearly stimulated the development of Leydig cells and many Leydig cells were present 35 days after administration of EDS. This indicates that repopulation of Leydig cells can occur only in the presence of LH. This was confirmed by the experiments performed with hypophysectomized rats which showed that in testes from untreated and FSH-treated rats leydig cells were still absent 35 days following EDS, whereas Leydig cells active in testosterone production were present in hCG-treated rats. Hence, the new population of Leydig cells originates from an hCG(LH)-dependent precursor cell and is independent of FSH, prolactin and other pituitary hormones. Kerr et al. (29) have demonstrated that hCG binds to the indeterminate connective tissue cells, which makes this cell type a likely candidate to be the precursor cell for Leydig cells.

Our results demonstrate that the cytotoxic effects of EDS on testes from sterile rats differs from those on testes from normal rats. In sterile rats occasionally single cells with the appearance of Leydig cells were still present between 4 and 9 days after EDS. Yet, at day 3 the plasma testosterone concentration was extremely low which means either that the remaining Leydig cells were no longer active in steroid production or that the number of these cells was too low to produce substantial amounts of testosterone. Apparently in sterile rats a few Leydig cells are resistant to the destructive effects of EDS, unlike in normal rats where no Leydig cells could be identified at day 7 after administration of EDS. Furthermore, the repopulation was much faster in the sterile rats. In normal rats 14 days after EDS almost exclusively single and paired Leydig cells were present, whereas in the sterile testes already clusters of more than 10 Leydig cells were present. Moreover in some sterile rats steroidogenic active cells could be isolated at day 7, whereas at day 14 all cell preparations from sterile rats showed steroid production. In normal rats this could be demonstrated only on day 21.

It is not yet possible to explain the difference in depletion and rate of repopulation between normal and sterile rats. It might be that the microenvironment of the interstitial tissue in sterile rats is different from normal rats because of an altered Sertoli cell function (15). There are indications from *in vitro* studies that local factors may play an important role in the cytotoxic effects of EDS, since EDS administration *in vitro* inhibits the LH dependent steroid production but does not affect the cell viability of isolated Leydig cells during the first 24 h (30). A difference in microenvironment may therefore explain the survival of some Leydig cells in testes from sterile rats in contrast to testes from normal rats where all Leydig cells were destroyed. Another possibility may be that the Leydig cells in normal and sterile rats have undergone different patterns of

differentiation. Previous studies (3) have demonstrated that Leydig cells from immature rats are resistant to EDS. The few Leydig cells remaining in testes from sterile rats may therefore represent Leydig cells with some "immature" properties.

It seems unlikely that these few cells are responsible for the faster repopulation, since from day 4 to day 9 these cells remained rare and only occurred as single cells and there were no signs of proliferation of these cells during this period. Only at day 11 more Leydig cells, some in groups of 4 and some as single cells, were observed indicating that precursor cells have differentiated into Leydig cells. The difference between the rate of repopulation in normal and sterile rats after administration of EDS is probably caused by either a higher number of Leydig cell precursors in sterile rats, which may be the result of an altered microenvironment or of the higher levels of the gonadotrophic hormones already before the EDS treatment or by a higher rate of proliferation of these cells in sterile rats, which may be induced by the higher levels of the gonadotrophic hormones soon after EDS treatment, possibly in combination with an altered microenvironment. Since the precursor cells are unknown, it is impossible to indicate which alternative is correct.

It has been reported that during sexual maturation in addition to LH the presence of FSH is required for normal development of Leydig cells (8,9,10). Experiments from Kerr et al. (11,2) even suggest that FSH alone enables the differentiation of Leydig cells, whereas LH alone is ineffective. This is in clear contradiction with our observations on the development of Leydig cells in mature rats after EDS treatment, where LH alone induces the differentiation of Leydig cells. The independence of FSH, however, does not exclude an essential role of local factors produced by the Sertoli cell, but in mature rats these factors may be produced independent of pituitary function. A similar situation has been described for the development of germ cells

which is dependent on Sertoli cells. In immature rats FSH is required for the initiation of spermatogenesis, but is not longer needed for the maintenance of spermatogenesis in mature rats (31,32).

During sexual maturation Leydig cell differentiation takes place in parallel with many other developmental changes in the tubular compartment, such as differentiation of Sertoli cells. Under these conditions it is very difficult to study the hormonal regulation of Leydig cell development. The EDS-treated mature rat, therefore, offers a more simple model to study the development and differentiation of Leydig cells, because the surrounding cells in testes from these rats had already reached a differentiated stage.

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