CHEMICAL DISRUPTION OF SPERMATOGENESIS.

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DECLARATION:

Except where acknowledgement is made in the text by reference, the experiments described in this thesis were the unaided work of the author. All experiments were performed in the Medical Research Council Reproductive Biology Unit in Edinburgh, Scotland, or at I.C.I. plc, Central Toxicology Laboratory, Alderley Park, Cheshire.

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CONTENTS.

ABSTRACT.

Over the past decade many publications attest to the substantial increase in public and scientific concern regarding the potential of industrial and environmental chemicals to interfere with male reproductive function. Spermatogenesis, (the process of sperm formation), depends on a series of complex biological interactions which can be interrupted at various stages by chemical insult. Recently, a number of these chemicals have been used experimentally to directly disrupt testicular function to provide an insight into the processes involved in normal spermatogenesis. Based on these investigations the primary objective of the work in this thesis was to study the time course of development of testicular lesions, their stage specificity, and subsequent recovery of normal spermatogenesis using morphological and functional characteristics of function *in vivo* and *in vitro*. The chemicals used in these studies included *meta* dinitrobenzene (mDNB), nitrobenzene (NB), the isomers of mononitrotoluene (mNT) and 2-methoxyacetic acid (MAA).

Within 24h of a single oral administration of mDNB or NB to rats there was a progressive decrease in testicular weight and increase in serum follicle stimulating hormone (FSH) levels coincident with widespread germ cell degeneration, as determined by histological examination of the testis. The degree of disruption induced by mDNB and its reversibility were time - but not stage - dependent. NB however had a more specific effect causing the initial degeneration of pachytene spermatocytes and subsequently more extensive germ cell loss involving round spermatids at a number of stages. During the recovery of spermatogenesis a considerable change was observed in the frequency of occurrence of spermatogenic stages in comparison to controls. These changes remained significantly different up to at least 70 days post-treatment. Epididymal content was also assessed under histological examination after NB administration as a further indirect index of testicular disruption. Results demonstrated that degenerating germ cells and giant bodies were present in the epididymis at a number of time intervals post-treatment.

The isomers of mNT induced small but statistically significant dose-dependent decreases in testicular weight. Spermatogenesis in these animals remained qualitatively normal, so detailed histological studies were not undertaken.

Further investigation into the effects of the testicular toxicants listed above was performed *in vitro* using Sertoli cell monocultures and Sertoli cell-germ cell co-cultures. mDNB, NB and the isomers of mNT produced dose-dependent increases in germ cell exfoliation (from co-cultures). mDNB and NB

also produced histological changes in culture including Sertoli cell vacuolation and phagocytosis of germ cells, and induced dose-dependent increases in the secretion of lactate and pyruvate, two established indices of Sertoli cell function *in vitro*.

Sertoli cells secrete over 100 different proteins, only a handful of which have been characterised and a role in spermatogenesis proposed. One of these proteins is inhibin. The potential of inhibin secretion as a further index of Sertoli cell function (or dysfunction) in monocultures and co-cultures has been investigated. mDNB, NB, 2-mNT and 3-mNT all induced dose-dependent alterations in the secretion of inhibin *in vitro*.

Historically, FSH has been postulated to control inhibin secretion in a classical negative feedback manner. Recent evidence obtained *in vivo* and in co-culture suggests that its secretion may be controlled by specific type(s) of germ cells. Seminiferous tubule cultures represent a potentially more useful culture system to investigate this further. They present a unique opportunity to study Sertoli cell-germ cell interactions under more physiological conditions since the integrity of the seminiferous epithelium is maintained *in vitro*. Previous investigators have demonstrated that MAA specifically destroys pachytene spematocytes *in vivo* and this specificity of action was utilised to study which germ cell-Sertoli cell interactions control the secretion of inhibin. At selected times after MAA treatment when specific complements of germ cells were absent or grossly depleted, seminiferous tubules were isolated and cultured to measure inhibin secretion under basal or stimulated conditions. The results demonstrate that elongate spematids are the primary germ cell type which exerts major control over inhibin secretion under both basal and FSH-stimulated conditions.

In conclusion, these studies demonstrate: Firstly that these compounds can be used as a means to investigate normal spermatogenesis. Secondly, effects of these compounds on spermatogenesis can be demonstrated both *in vivo* and *in vitro*. Finally, that the seminiferous tubule culture technique represents a more physiological approach with which to investigate *in vivo-in vitro* comparative toxicity with respect to chemically induced disruption of spermatogenesis. These studies are among the first to demonstrate the possibility of developing germ cells modulating Sertoli cell function with respect to inhibin secretion mediated through the release of paracrine factors. Further investigations using this seminiferous tubule culture model and other techniques, specifically the use of 2-dimensional polyacrylamide gels may aid in the identification and investigation of paracrine factors between Sertoli cells and germ cells which may modulate the production of inhibin.

CHAPTER 1.

LITERATURE REVIEW.

1.0. INTRODUCTION.

Over the past decade growing public and scientific concern has focused attention on a number of chemicals used in household products and industrial processes which have been identified as testicular toxicants. Accidental exposure of humans to a number of these compounds has resulted in reduced fertility whilst exposure of laboratory animals to many others causes specific disruption of testicular function (Mattison, 1983; Bernstein, 1984; Dixon, 1985; Philips *et al*, 1985; Waller *et al*, 1985; Thomas, 1986; Drife, 1987; Lamb & Foster, 1988; Schardein, 1988). Methods used to identify and evaluate possible testicular toxicants however are often insensitive to highly specific testicular disruption which may result in infertility. These tests are also expensive. Tests used to identify possible toxicants of the male reproductive system rely on: 1) Morphological evaluation of testicular tissue following toxicant injury. 2) Fertility assessment in breeding studies. 3) The application of toxicants to testicular cell cultures. Whilst these tests have identified a number of toxicants a sensitive biochemical marker of testicular function is required to improve the assessment of compounds on testicular function.

The problems associated with evaluating the effects of possible testicular toxicants are compounded by our incomplete understanding of the basic processes involved in normal spermatogenesis. Experimental approaches used to examine normal testicular physiology have followed two avenues, *in vitro* or *in vivo* investigations. A number of techniques have been used to perturb testicular function *in vivo* to compare normal and disrupted spermatogenesis, these include heat treatment, X-irradiation and cryptorchidism; whilst *in vitro*, testicular cells are cultured in homogenous or heterogeneous preparations. Recently a number of known testicular toxicants have been utilised as biochemical tools to examine both *in vivo* and *in vitro* the processes involved in spermatogenesis.

The aims of the studies presented in this thesis were two fold: Primarily detailed assessment of the effects of known and/or suspected testicular toxicants and an evaluation of their usefulness for investigating Sertoli cell physiology and the role of Sertoli cells in spermatogenesis, and secondly, as a possible "spin off" from the above, to identify a possible biochemical marker of Sertoli cell function which could be used in reproductive toxicity testing *in vivo* and *in vitro*.

Studies were performed in the rat since it has been the animal used most frequently as an *in vivo* model in reproductive toxicology. In terms of general pharmacology, endocrinology and reproductive physiology, the rat is the

species most thoroughly evaluated. Whilst anatomical, physiological and biochemical differences between the rat and man do exist, the rat is still the preferred species for most toxicity studies. In addition, cultures derived from rat testicular tissue were used to examine in more detail the effects of compounds *in vitro*.

Before discussing the particular toxicants used in this thesis it is pertinent to review the structural organisation of the testis, its physiology and the complex role played by endocrine hormones and locally produced paracrine factors in spermatogenesis. Emphasis is placed on the Sertoli cell and developing germ cells and their interactions within spermatogenesis since the toxicants utilised throughout this thesis, (with the exception of one), are all known and/or suspected Sertoli cell toxicants, the exception being a suspected germ cell toxicant.

1.1. STRUCTURE OF THE TESTIS.

The testes are bilateral organs which in most mammals, including man and the rat, are located within the scrotal sac external to the body and maintained at a temperature of 32-34°C. The role of the testis is two-fold; firstly the production of gametes capable of fertilising an oocyte and secondly the production of testosterone, the 'driving force' behind spermatogenesis and responsible for the development of male secondary sexual characteristics and the sex drive. To perform these functions the testes has a highly complex structural organisation of cells, each with a specialised function and dependent overall on endocrine support.

The mammalian testis may be divided into two compartments; the avascular seminiferous tubules and the fluid filled interstitium. Seminiferous tubules are long convoluted cylindrical structures which contain Sertoli cells and various generations of differentiating germ cells which together comprise the seminiferous epithelium. Within the centre of the seminiferous tubule is a fluid filled lumen. The seminiferous tubules are connected at either end to the rete testis which channels gametes sloughed from the epithelium and transported along the tubule through the efferent ducts to the epididymis for maturation and storage (see Fig 1.1). The tubules are surrounded by a layer of peritubular myoid cells, which is inturn surrounded by the interstitium. The interstitium is composed of sparse, loose connective tissue interspersed with Leydig cells, macrophages, non-myelinated nerves, vascular and lymphatic vessels and interstitial fluid (IF) - a filtrate of serum (Setchell & Sharpe, 1981) which bathes the seminiferous tubules: (For reviews see Ross, 1967; Fawcett *et al*, 1973;
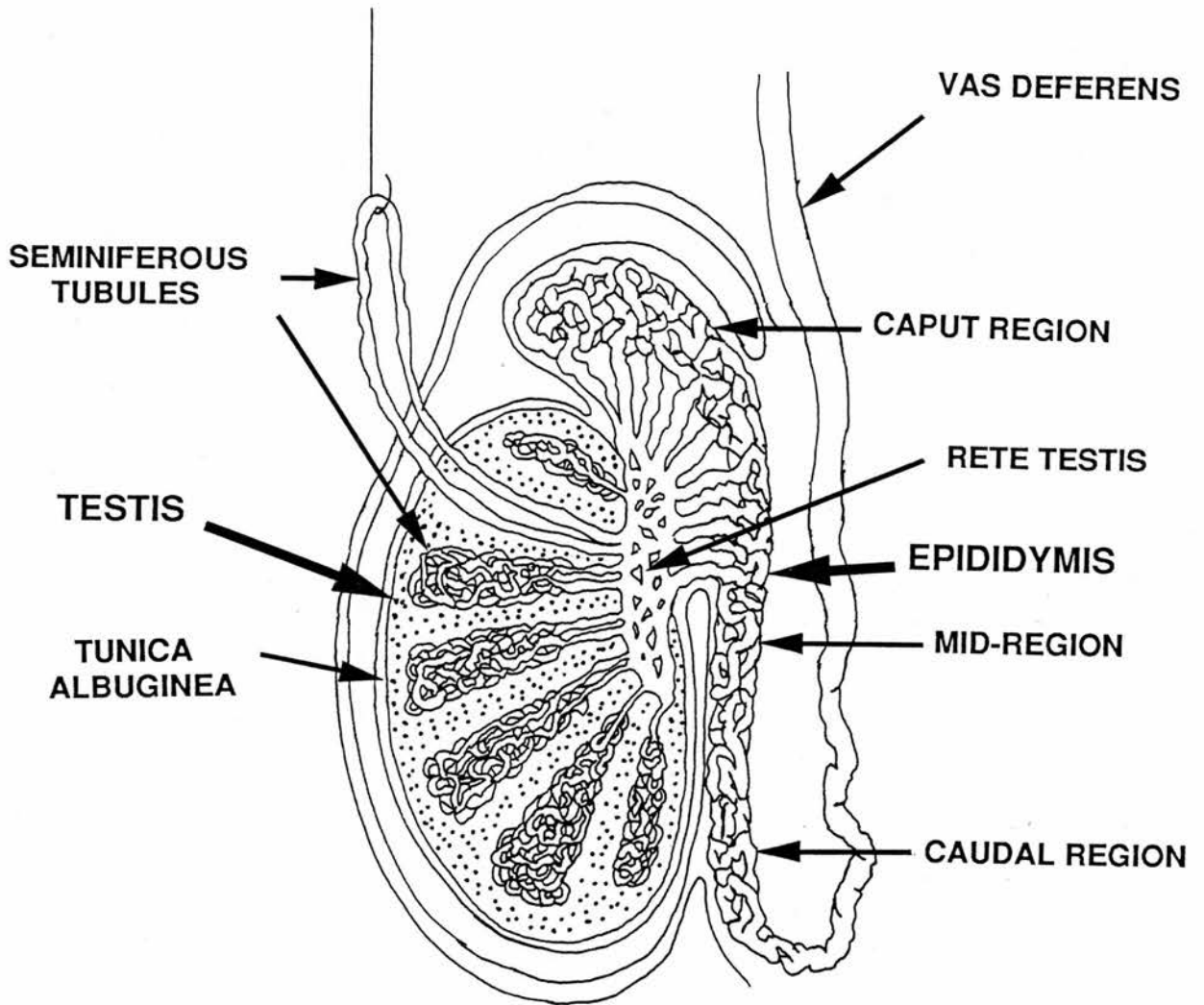
Fig 1.1. Section through the adult human testis
and epididymis shown structures.

Christensen, 1975; Clark, 1976; Connell & Connell, 1977 and Ewing & Zirkin, 1983). These structures are enclosed within the tunica albuginea, which forms the capsule of the testis.

1.1.1. Organisation within the seminiferous tubule.

Sertoli cells are columnar in shape with extensive cytoplasm extending from the basement membrane to the lumen of the seminiferous tubule between developing germ cells. The various generations of germ cells are not distributed randomly but are arranged in specific cellular associations or stages. Each stage is composed of four or five generations of germ cells at different points in their development including one or two generations of spermatogonia, spermatocytes and spermatids.

Leblond and Clermont (1952) first categorised these specific cellular associations in the rat seminiferous tubule into fourteen stages of spermatogenesis (see Fig 1.2). That is, if one cuts the tubule in cross section and puts the matching cross sections into piles, there would be 14 piles or 14 cell associations which keep recurring. From another perspective, if one watched a particular portion of the tubule over 12.8 days, each of the fourteen stages would appear in sequence. Stages therefore follow each other in a radial manner consecutively along the length of each seminiferous tubule i.e. stage VII is followed by stage VIII and preceded by stage VI. One complete sequence of stages I to XIV is known as a spermatogenic wave (Fig 1.2) and represents the succession of cellular associations along the length of the tubule. In contrast, the spermatogenic cycle is the succession of different stages in time. The duration of individual stages is constant but varies between stages i.e. stage III lasts 6h whilst stage VII lasts 62.8h. One complete sequence of stages I-XIV takes 12.8 days. Modifications to the order of stages do exist i.e. when the stage sequence is reversed - I-II-III-IV-III-II-I etc, however the underlying theme is maintained so that an individual stage is one stage ahead or behind its adjacent neighbour (Fig 1.2). Thus a progressive wave of maturation passes along the tubule.

The discovery that the process of spermatogenesis could be categorised into specific cell associations has been instrumental in aiding our understanding of the effects of toxicants on the testis. Since we know what cell associations should be present in each stage of spermatogenesis, tubules missing cell types or cells in the wrong stage would indicate altered spermatogenesis. For example, mature step 19 elongate spermatids are normally released in stage VIII (Russell, 1980). If they are still present in what appears by examination of

The Spermatogenic Cycle
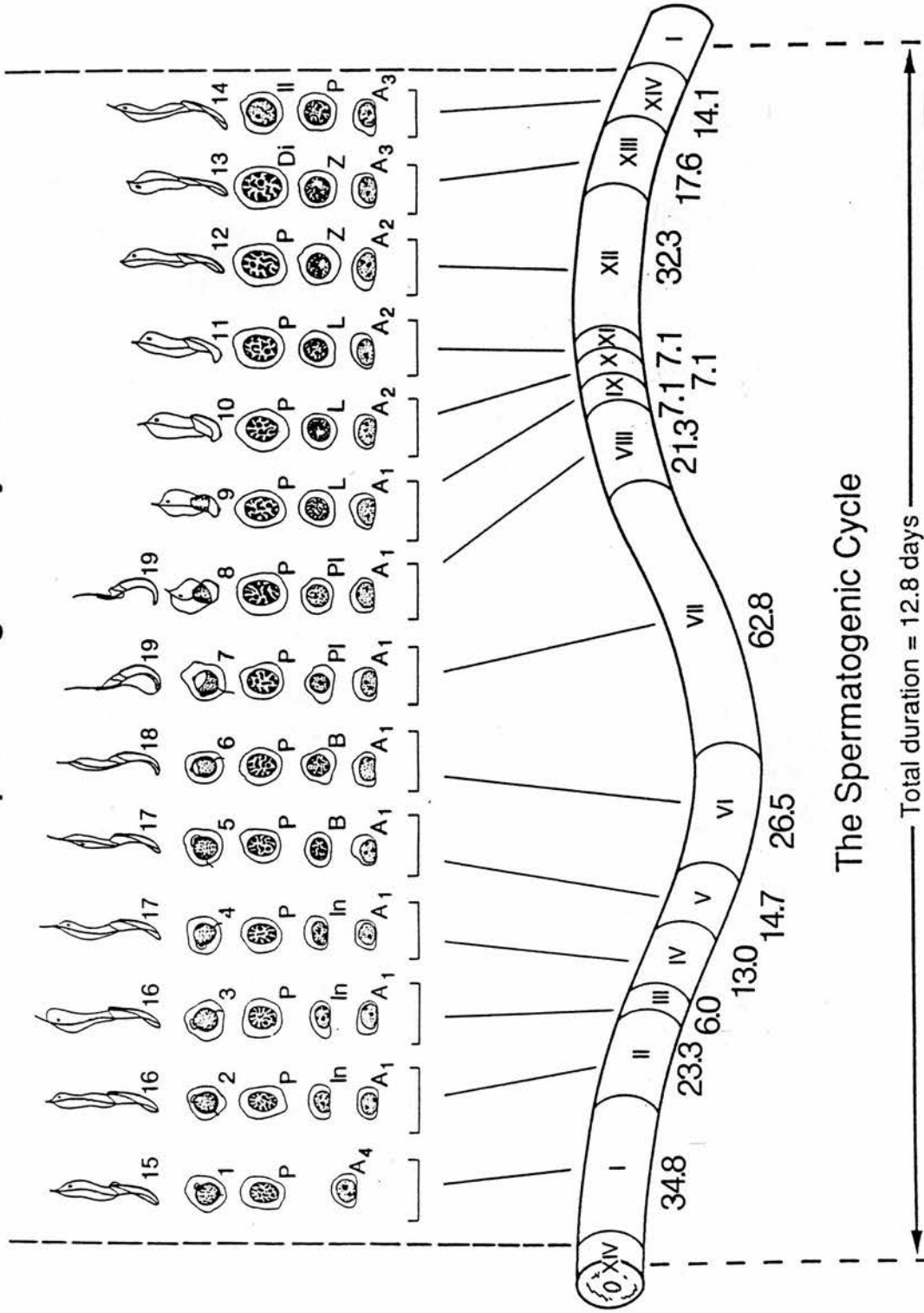
Fig 1.2. Diagramatic representation of the spermatogenic cycle in the rat to illustrate the particular association of the germ cell types present in the seminiferous epithelium at each stage of the cycle and the sequential distribution of these stages along a segment of seminiferous tubule. Different stages last for different (but fixed) periods of time (shown under each stage in hours), and the length of each stage in the tubule segment has been drawn in proportion to its duration to illustrate this. It should be noted that these proportions do not correspond to the length of the tubule occupied by the different stages in reality, since this is extremely

the other cell types to be stage IX tubules, then it is evident that something has disrupted spermiation (the release of spermatozoa). Also, one can quantitatively determine if all stages are present or quantitatively count the number of each cell type in each stage to examine for effects of toxicants on the number of germ cells.

1.1.2. Germ cell differentiation.

The process of germ cell differentiation takes place within the seminiferous tubule and commences with six successive divisions of undifferentiated type A spermatogonia. These cells divide mitotically to maintain both type A_O stem cells and to provide cells which initiate each wave of spermatogenesis. The process is repeated cyclically at a fixed interval of 12.8 days in the rat. This represents the time between two successive stem cell divisions and this is termed the duration of the spermatogenic cycle (Clermont, 1959). Six mitotic divisions occur to form A_1, A_2, A_3, A_4, intermediate and type B spermatogonia, which then further divide to produce preleptotene spermatocytes. These go through DNA replication at the start of meiotic prophase to form primary spermatocytes. During the following leptotene, zygotene, pachytene and diplotene spermatocyte stages the homologous pairing of chromosomes is initiated and completed. The pachytene spermatocyte stage is the longest, after which the first meiotic division occurs yeilding secondary spermatocytes at stage XIV. These pass rapidly through the second meiotic division (minus DNA synthesis) and form haploid round spermatids. These cells then pass through a phase in which dramatic structural transformations occur, resulting in the formation of elongate spermatids (Russell, 1980). Theoretically this cascade of cell divisions gives rise to a clone of 256 elongate spermatids; in practice the number is less because of germ cell degeneration (Roosen-Runge, 1973). The process of mitosis, meiosis, spermiogenesis (development of spermatids) and spermiation (release of elongated spermatids into the lumen) comprises of 4.5 spermatogenic cycles and takes 53.2 days in the rat (Clermont, 1959).

As the process of germ cell maturation takes place germ cells are translocated from the basement membrane of the seminiferous tubule towards the lumen resulting in the gradual displacement of successive generations of germ cells. Fully mature step 19 elongate spermatids (i.e. spermatozoa) are released into the lumen to be transported, together with the fluid in the lumen, to the epididymis, where the spermatozoa mature and aquire fertilising ability. The

Sertoli cell performs a number of essential functions in the control of germ cell differentiation which are discussed in greater detail below.

1.1.3. The Sertoli cell.

First described by Sertoli in 1865, the Sertoli cell is a highly differentiated cell which plays a fundamental role in spermatogenesis. By virtue of its position within the seminiferous tubule the Sertoli cell can create a specialised environment for germ cell differentiation and maturation. In order to perform these functions Sertoli cells have a number of key structure/function features. These include: a) Numerous cytoplasmic processes which envelop and inter-digitate between developing germ cells (except spermatogonia) to provide both physical support and communication between adjacent Sertoli cells throughout the seminiferous epithelium (Russell, 1980; 1987). b) The ability to translocate germ cells from the basal lamina (basement membrane) through the epithelium to the lumen between adjacent Sertoli cells (Russell, 1977; 1980). c) The ability to phagocytose residual bodies (i.e. residual germ cell cytoplasm remaining after spermiogenesis), or abnormal germ cells (Russell & Clermont, 1977) or degenerating germ cells (Blackburn et al, 1988). d) Sertoli cell tight junctions (Fawcett, 1975; Russell, 1978). These highly specialised structures are located at the base of each Sertoli cell between adjacent Sertoli cells. This physical barrier isolates germ cells at and beyond the prophase of meiosis in the adluminal compartment of the seminiferous tubule interposed between adjacent Sertoli cells and separated from spermatogonia in the basal compartment (Fawcett 1974; 1975). In the rat tight junctions form at around 15 days of age along with the development of primary spermatocytes (Dym & Fawcett, 1970; Vitale et al, 1973) and the cessation of Sertoli cell mitotic activity (Steinberger & Steinberger, 1971). The existence of the barrier was suspected from indirect evidence that the composition of seminiferous tubule and rete testis fluid was different from blood plasma and testicular IF (Setchell, 1969). Confirmation of the existence of the barrier came from a technique involving electron microscopy and electron dense extracellular markers lanthanum and horseradish peroxidase (Russell, 1978). This barrier is commonly known as the "blood testis barrier", although in many respects this is a misnomer since it exists towards the base of the seminiferous tubule rather than, as is suggested by the name, at the level of the blood vessels (see Fig 1.3).

The primary function of the barrier is to exclude a variety of substances and to maintain a specific ionic and hormonal intra-tubular environment suitable for meiosis and maturation of post-meiotic germ cells
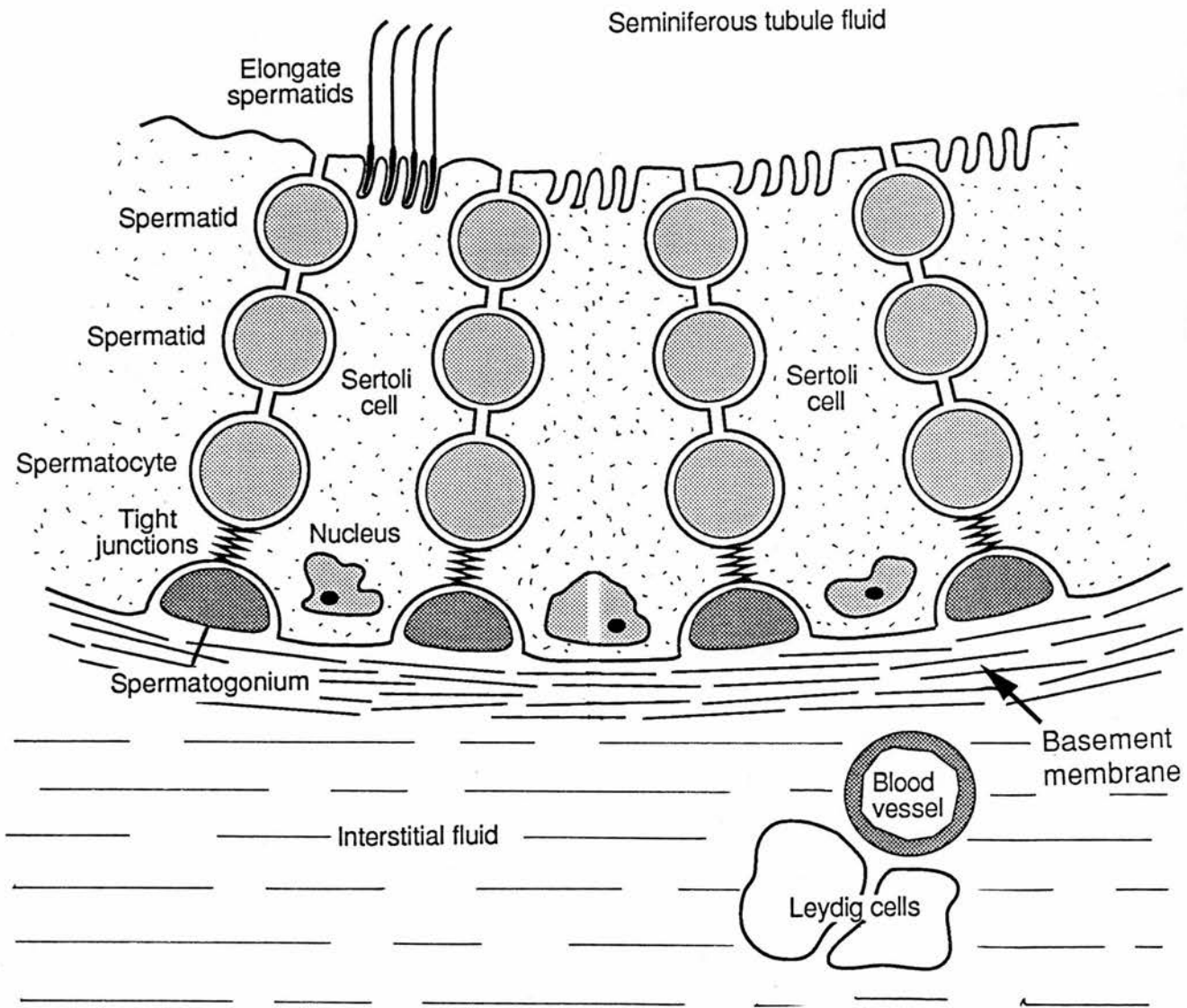
Fig 1.3. Diagramatic representation of a segment of seminiferous epithelium at stage VII of the spermatogenic cycle and the adjacent intertubular space.

(Setchell, 1980). For example high potassium and bicarbonate concentrations and low sodium and chloride levels are maintained within the tubule in comparison to blood plasma and IF levels (Setchell & Waites, 1975; Waites & Gladwell, 1982). The barrier has a secondary role in preventing an immune response. The developing secondary spermatocytes are haploid and are therefore classified by the immune system as 'foreign' cells (Millette & Bellve, 1977; O'Rand & Romrell, 1977). In order to prevent the formation of antibodies against germ cells the Sertoli cell barrier is impermeable to cells of the immune system.

In addition to the above structural features the Sertoli cell has a secretory role to play in spermatogenesis. Sertoli cells synthesis seminiferous tubule fluid (Setchell, 1980), and regulate spermatogenesis at the metabolic level by producing energy substrates for germ cells and by synthesising and secreting specific proteins which are thought to control not only spermatogenesis at the paracrine level (discussed later) but to modulate the functions of other intra-testicular cells including Leydig cells and peritubular cells (discussed later). Sertoli cells also have an endocrine role to play because they produce inhibin in response to FSH stimulation which is thought to feed back at the level of the pituitary to regulate FSH secretion (discussed later).

We are only just beginning to be understand the nature and control of these secretions and their possible roles in spermatogenesis: (For reviews see Fawcett, 1975; Steinberger & Steinberger, 1976; Russell, 1980; Mather *et al*, 1985; Saez *et al*, 1985; 1987; Tindall *et al*, 1985; Sanborn *et al*, 1987 and Bardin *et al*, 1988).

1.1.3.1. Sertoli cell secretions.

To date primary cultures of Sertoli cells have been shown to secrete 84 proteins (Cheng *et al*, 1986; Perrard-Sapori *et al*, 1986), and, using seminiferous tubules Wright (1983) has identified less than 100. These include endocrine hormones, growth factors, enzymes, matrix components, a host of other factors whose functions are as yet unknown and energy substrates for germ cells. Those secretions identified include; androgen binding protein (ABP) (Hansson *et al*, 1975; Bardin *et al*, 1981; Ritzen *et al*, 1981), inhibin (de Jong & Robertson, 1985), seminiferous growth factors (Feig *et al*, 1980; 1983; Brown *et al*, 1982; Holmes *et al*, 1986), somatomedin (or insulin like growth factor I; Hall *et al*, 1983), transferrin (Skinner & Griswold, 1983), ceruloplasmin (Skinner & Griswold, 1983), lactate and pyruvate (Jutte *et al*, 1983), retinol binding protein (Huggenvik & Griswold, 1981; Carson *et al*, 1984),

plasminogen activator (Lacroix *et al*, 1977; Vihko *et al*, 1984), clusterin (Fritz *et al*, 1983; Blaschuk *et al*, 1984; Tung & Fritz, 1985), cyclic proteins (Wright *et al*, 1983; Wright & Luzavaga, 1986; Wright, 1988) and testibumin (Cheng & Bardin, 1986). A number of these secretions have been utilised in an attempt to monitor Sertoli cell function and possible dysfunction following heat treatment, X-irradiation, cryptorchidism and toxicant pertubation *in vivo* and *in vitro*.

Within the context of this review only a few examples are used to examine the role of Sertoli cell secretions in spermatogenesis, (for further examples the reader is referred to the reviews by Griswold, 1988 and Bardin *et al*, 1988).

ABP was the first Sertoli cell protein to be identified and studied extensively (French & Ritzen, 1973; Hansson *et al*, 1975). Its secretion is stimulated by both FSH and testosterone *in vivo* in the immature rat. The ABP secreted into the tubule lumen is transported along with spermatozoa to the epididymis. As it moves through the epididymis ABP is apparently taken up by the epididymal epithelial cells as minimal amounts are detectable in the lumen of the vas deferens. The role (if any) of ABP in spermatogenesis is unknown, however a numer of roles have been postulated: 1) It has a high binding affinity for testosterone and dihydrotestosterone, therefore it has been proposed as a carrier protein within the Sertoli cell. 2) It may maintain the high concentrations of androgens within the seminiferous tubule and/or epididymis. 3) It may provide androgens to the androgen regulated epithelial cells which line the epididymis as well as playing a role in the androgen sensitive stages in spermatogenesis. Although data supporting all of these possible functions is limited, they are all consistent with the steroid-binding activity exhibited by ABP (Lobl, 1981; Campo *et al*, 1982). Measurement of serum and interstitial fluid ABP have been proposed to be a valuable marker of Sertoli cell function even though the relationship between ABP and Sertoli cells is poorly understood (Gunsalus *et al*, 1981; Bartlett & Sharpe, 1987).

Sertoli cells also secrete a number of classical hormones including anti Mullerian hormone and inhibin, which have a high degree of homology. The former is produced in neonatal Sertoli cells and stimulates the regression of the Mullerian ducts in the male foetus (Josso *et al*, 1977; Donahoe *et al*, 1982). Secretion of the latter is stimulated by pituitary FSH and inturn inhibin is thought to negatively regulate the synthesis and secretion of FSH. Inhibin is one of the most studied of the Sertoli cell secretory products, see reviews of de Jong, 1987; 1988; McLachlan *et al*, 1988; Ying, 1988 and de Kretser *et al*, 1989). Whilst the initial evidence of a water soluble testicular regulator of

pituitary function was presented over 50 years ago (McCullagh, 1932), it was only in the last decade that Steinberger and Steinberger (1976) showed that the Sertoli cell was the site of production of a factor which could selectively inhibit FSH release from cultured pituitary cells. It is only recently that radioimmunoassay have been developed for inhibin measurement (Mc Lachlan *et al*, Sharpe *et al*, 1988). The fully processed form of inhibin has a molecular weight of 32 kDa, composed of two dissimilar subunits linked by disulphide bridges. These subunits are known as α and β and can be linked in various combinations. In addition, two forms of unglycosylated beta (β) subunit exist denoted as β-A and β-B (Ling *et al*, 1985; Mason *et al*, 1985; 1986; Stewart *et al*, 1986). Inhibin-A is thus formed by the dimerization of α and β-A subunits, and inhibin-B from α and β-B subunits. The application of immunohistochemistry for the localisation of inhibin-like peptides and in-situ hybridisation for the localisation of the mRNA's encoding for the α-subunit have confirmed that Sertoli cells are a source of testicular inhibin both *in vivo* and *in vitro*, and that the secretion of inhibin is stimulated by FSH and cAMP (Lincoln *et al*, 1989) in primary Sertoli cell cultures in a dose-dependent manner (Ultee van Gessel *et al*, 1986; Biscak *et al*, 1987; Morris *et al*, 1988) and in cultures of isolated seminiferous tubules (Gonsalus *et al*, 1988; Sharpe *et al*, 1988). Recent evidence suggests that adult Leydig cells produce inhibin (Risbridger *et al*, 1989; Maddocks & Sharpe, 1989). The physiological significance of this is unclear, however Maddocks and Sharpe (1989) have demonstrated that, in the adult rat, the proportion of testicular/blood inhibin emanating from the Leydig cell is negligible.

It is now established clearly that FSH stimulates inhibin production by the Sertoli cell and that this inhibin acts on the pituitary to negatively regulate FSH secretion under a number of experimentally induced situations *in vivo* and in the normal pre-pubertal rat (Rivier *et al*, 1988) in addition to the negative feed back of testosterone on FSH (discussed later). However the role of inhibin in the adult animal remains unclear. It is possible that inhibin may be a paracrine factor in testicular function. Studies by van Dissel-Emiliani *et al* (1989) have shown that inhibin administration reduces spermatogonial number in testes of adult mice and chinese hamsters. They have demonstrated that the numbers of A_4, intermediate and B spermatogonia decrease significantly by comparison to control following intraperitoneal (IP) or intratesticular (IT) injections of bovine follicular fluid extracts into mice and hamsters respectively, but that the numbers of undifferentiated spermatogonia do not change. Partially purified inhibin from Sertoli cell conditioned media had the same effect. In cases of IP injection of

inhibin, peripheral FSH levels were significantly depressed post-treatment suggesting that FSH might be a modulator of the actions of inhibin on spermatogenesis. However in experiments in which unilateral IT injections of inhibin were administered, effects were confined to the injected testes suggesting that the effects on the seminiferous epithelium were not FSH-mediated.

Sertoli cells also play an important role in providing energy substrates for the developing germ cells in the form of lactate, pyruvate and α-keto acids. These are critical for the maintenance of germ cells. Sertoli cells have a high rate of glycolysis and secrete lactate and pyruvate in the ratio of approximately 4:1. Secretion of both lactate and pyruvate can be stimulated by FSH *in vitro* (Jutte *et al*, 1983). Additionally, studies on isolated germ cells have indicated that these cells cannot maintain their ATP levels if supplied with glucose, but can do so on addtion of lactate and to a lesser extent pyruvate (Jutte *et al*, 1981; 1982; Mita *et al*, 1982; Grootegoed *et al*, 1985). Similarly, aminotransferases, which convert branch chain amino acids to α-keto acids, are localised in Sertoli but not germ cells. Thus, α-keto acids may be utilised as an energy source via the germ cell specific lactate dehydrogenase C-4 isoenzyme (LDH-C4) which converts α-keto acids to α-hydroxy acids, thus shuttling reduced equivalents into germ cell mitochondria (Foster, 1988). All haploid germ cells possess LDH-C4 (pachytene spermatocytes, round and elongate spermatids) and it has been proposed as a possible biochemical marker of testicular function *in vivo*. Spermatogenic cell disruption could result in leakage of this enzyme into interstitial fluid and seminiferous tubule fluid, eventually reaching the blood supply (Goldberg, 1977). The detection of this isoenzyme in blood would therefore indicate testicular disruption. However its use has been severely limited because the serum levels of LDH-C4 following testicular disruption were not high enough or tests not sensitive enough to detect LDH-C4 in blood in addition to its lack of specificity (Foster - personnal communication).

Sertoli cells also secrete proteins normally found in serum: These include the metal binding proteins transferrin and ceruloplasmin, which bind iron and copper respectively, and acidic glycoproteins (Skinner & Griswold, 1983; Skinner *et al*, 1984). The role of testicular transferrin (similar to serum transferrin except for its carbohydrate content) and ceruloplasmin is to transport iron and copper from the serum to the adluminal compartment of the tubule, where they may be important for post meiotic germ cells which, because of their position behind the tight junction do not have access to plama derived transferrin and ceruloplasmin presumably present in interstitial fluid. The demonstration of transferrin receptors on Sertoli cell s, spermatocytes and spermatids (*in vitro*)

and the transport of transferrin bound iron into Sertoli cells and spermatids (*in vitro*) is consistent with this hypothesis (Toebosch *et al*, 1987). Both ceruloplasmin and transferrin have also been demonstrated to be produced stage-dependently (Wright *et al*, 1983-discussed later).

Several stimulatory growth factors are also secreted by Sertoli cells (Bellve & Feig, 1984; Holmes *et al*, 1984; Lamb *et al*, 1987). A putative stimulator of germ cell mitosis, termed seminiferous growth factor, has been purified and is being examined for its site and mechanism of action. A meiosis-stimulating and a separate meiosis-inhibiting factor produced by Sertoli cells appears to be secreted differentially at certain stages of the spermatogenic cycle (Parvinen, 1982; 1986).

Other secretions of the Sertoli cell include enzymes such as plaminogen activator and an α-lactalbumin-like protein. The former is a specific protease responsible for the conversion of plasminogen to plasmin. This enzyme is thought to be involved in the translocation of germ cells within the seminiferous epitheilium, for the release of spermatids during spermiation (Lacroix *et al*, 1979) and the transfer of preleptotene spermatocytes through the Sertoli cell tight junctions at stage VII when differentiated spermatogonia enter meiotic prophase and become 'foreign' to the immune system (Russell, 1977; Lacroix *et al*, 1981; Parvinen, 1982). Further evidence for its role comes from the stage specific culturing of seminiferous tubules taken from X-irradiated animals in which preleptotene spermatocytes are absent. The peak in plasminogen activator activity normally associated with stages VII and VIII is ablated, whilst only minor changes occur when other classes of germ cells are depleted (Vihko *et al*, 1984; Parvinen *et al*, 1986 - discussed later).

Hamilton (1984) first reported the presence of lactalbumin-like activity in epididymal fluid. It was suggested that this molecule was made by Sertoli cells and recently α-lactalbumin has been identified in Sertoli cell culture medium (Skinner & Fritz, 1986). However its role in spermatogenesis is unknown.

1.1.3.2. Bidirectional secretion by the Sertoli cell.

Recently it has become apparent that a number of Sertoli cell products are secreted in a bidirectional manner (Sharpe, 1988), i.e. secretions occur via the apical and basal aspects of the Sertoli cell into seminiferous tubule fluid and interstitial fluid. Several recent findings suggest that this may be an important physiological function of Sertoli cells. In the adult rat, 80% of the ABP secreted by the Sertoli cell is secreted apically into the seminiferous tubule

fluid whilst the remaining 20% is secreted into the interstitial fluid (Gunsalus *et al*, 1980; Mather *et al*, 1983). This could be due to "leakage" or simply be unimportant. However, evidence suggests that the bidirectional secretion of ABP may have a role. Secretion of ABP via the base of the Sertoli cell declines progressively during puberty in the rat (Gunsalus *et al*, 1980; Sharpe & Bartlett, 1987). The pattern of this decline is however not related to the formation of Sertoli cell tight junctions at around day 18 of age (Dym & Fawcett, 1970). In addition, if spermtogenesis is experimentally disrupted then ABP secretion into testicular interstitial fluid and thence blood is increased significantly (Gunsalus *et al*, 1980; 1981; Bartlett & Sharpe, 1987; Morris *et al*, 1987; 1988; Sharpe & Bartlett, 1987). Further evidence has demonstrated that the proportion of ABP secreted apically and basally differ in direct proportion to cases where spermatogenesis is disrupted specifically, with the loss of a particular complement of germ cells *in vivo* (pachytene spermatocytes and elongate spermatids) from the seminiferous epithelium (Pinon-Lataillade *et al*, 1986; Morris *et al*, 1987; Bartlett *et al*, 1988). This would tend to suggest that the germ cell complement modulates the direction of secretion of a Sertoli cell product *in vivo*. Further *in vitro* evidence has shown that selective germ cell types modulate the secretion of ABP by the Sertoli cell in culture (Galdieri *et al*, 1984; Le Magueresse *et al*, 1986; Le Magueresse & Jegou, 1986) (discussed in greater detail later).

Other Sertoli cell products which have been shown to be secreted in a bidirectional manner include inhibin (Baker et al, 1978), testibumin (Cheng & Bardin, 1986) and probably transferrin (Janecki & Steinberger, 1987). Direct evidence that inhibin is secreted bidirectionally has been demonstrated by Maddocks & Sharpe (1989). Measurement of inhibin levels in testicular venous, spermatic venous and peripheral venous blood and in interstitial fluid have demonstrated that in the adult rat inhibin is resorbed into the circulation via the mediastial venous plexus overlying the rete testis. Inhibin is therefore secreted apically from the Sertoli cell into seminiferous tubule fluid and absorbed at the plexsus. This situation however does not occur in immature rats in which inhibin secretion into interstitial fluid and thence blood is the predominant direction of secretion. Levels of inhibin can also be measured in interstitial fluid in adult rats, thereby suggesting that, like ABP, inhibin is secreted both apically and basally in the normal adult, but that the predominant route of secretion is apical. This situation however is further complicated by the demonstration that in adult rats Leydig cells produce inhibin (Risbridger *et al*, 1989), although this appears to be relatively insignificant (Maddocks & Sharpe, 1989).

With the introduction of dual compartment chambers and extracellular matrices on which to culture Sertoli cells further products are likely to be identified which are secreted in a bidirectional manner (Byers *et al*, 1986; Janecki & Steinberger, 1987). Such culture systems are discussed later.

1.1.3.3. Cyclical variations in Sertoli cell secretions.

With the overwhelming number of Sertoli cell secretions, the question to be addressed is: Do these vary cyclically in accordance with the spermatogenic cycle and if so what are their particular roles in spermatogenesis?

Parvinen reported in the early 1970's that when seminiferous tubules of the rat were observed under a stereomicroscope with transillumination, its light absorption varied with the stages of the cycle. When this technique was coupled with microdissection, a procedure to isolation specific sections of the tubule corresponding to particular stages of spermatogenesis was developed thereby allowing biochemical studies of each stage (Parvinen & Vanka-Pertulla, 1972; Parvinen, 1982; Parvinen *et al*, 1986). Using this technique a number of Sertoli cell products were found to be produced cyclically in accordance with the stages of the seminiferous tubule (discussed later). However, the first indication of changes in Sertoli cells correlating with stages of the seminiferous epithelium were apparent before transillumination techniques. Leblond and Clermont (1952) and Lacy (1960) using histological sections of the testis had shown that Sertoli cell morphology and lipid content varied during the cycle. Other changes identified since include cell volume (Caricchia & Dym, 1977), lipid morphology and position (Kerr & de Kretser, 1975) and general morphology (Kerr, 1988). Changes in the activity of certain enzymes during the spermatogenic cycle have also been demonstrated histochemically (Pertulla, 1972; Parvinen & Vartha 1978; Hilscher *et al,* 1979). Nonetheless, the majority of information concerning stage-specific changes in physiology have been from experiments utilising tubule segments isolated at specific stages of spermatogenesis. Stage specific changes identified in this manner include: Maximal ceruloplasmin secretion in stages IV to VI (Wright *et al*, 1983), Sertoli cell cyclic protein-2 secretion in stages V to VII (Wright *et al*, 1986), Sertoli cell aromatase inhibitor in stages VII to IX (Boitani *et al*, 1981), plaminogen activator activity in stages VII to IX (Lacroix *et al*, 1981; Vihko *et al*, 1987), production of meiosis inhibiting substances (stages VII to IX; Parvinen, 1982), acid phosophatase production (stages VII to XI; Parvinen, 1982), a high concentration of tubule nuclear androgen receptors (stages VII to XI; Isomaa *et al*, 1985), higher testosterone content in tubules at stages VII to VIII (Parvinen & Ruokonen, 1982), increased

cAMP phosphodiesterase activity (stages VIII to XI; Parvinen, 1982), increased ABP secretion (stages VIII to XI; Ritzen *et al*, 1982), increase in Sertoli cell secreted proteins (stages VI to IX; Wright *et al*, 1983; Shabanowitz *et al*, 1986), secretion of a tubule factor inhibiting Leydig cell testosterone production (stages VII to XI; Syed *et al*, 1986; 1987), increased Sertoli cell lipid content (stages IX to XIV; Kerr *et al*, 1984), increased transferrin secretion (stages IX to XII; Wright *et al*, 1983) and a change in the volume of the Sertoli cell (stage XI to XIII; Parvinen *et al*, 1986).

A few points to be highlighted are: Firstly, Sertoli cells undergo changes in morphology, lipid content, phagocytotic activity and enzyme content during specific stages of spermatogenesis. Thus, the metabolic activities of Sertoli cells appear to be synchronised with the proliferation, maturation, movement and release of germ cells. Secondly, FSH stimulation of Sertoli cell function, presumably via an increase in cAMP, varies with the stage of the cycle. There is an age-dependent decline in the ability of FSH to stimulate cAMP accumulation in cultured rat Sertoli cells and the overall response of adult Sertoli cells is quite low (Steinberger *et al*, 1978). However with the use of transillumination techniques it has been shown that the ability of FSH to stimulate cAMP accumulation is highest in stages I-IV, while stages VII and VIII are virtually insensitive to FSH. This is because: a) There are significant cyclical variations in the binding of FSH to its receptor. b) A stage specific variation in the ability of FSH to stimulate cAMP production exists. c) A variation in the activity of the cAMP degrading enzyme, phosphodiesterase, exists during different stages of the cycle (Parvinen 1982; Parvinen *et al*, 1986). Thirdly, there are cyclical changes in the concentration of testosterone in the tubule (highest in stages VII-IX and lowest in stages XIII-V). The maximal nuclear androgen receptor concentration is found in stages IX-I immediately following the maximal testosterone concentration. Fourthly, there are cyclically secreted proteins such as ABP (Sertoli cell), plasminogen activator (Sertoli cells and specific forms of spermatocytes), cyclic protein-2 (Sertoli cells) and acid phosphatase (Sertoli cells and specific spermatids forms) (Parvinen *et al,* 1986). The remarkable complexity of the system is further illustrated by the fact that FSH stimulation of plasminogen activator and ABP secretion from isolated seminiferous tubules is highest in stages VII-VIII which coincide with the lowest number of FSH receptors and cAMP response.

Studies by Bhasin *et al* (1989) have also demonstrated that there are stage-dependent differences in the expression of inhibin α and β-B subunits. These studies have demonstrated that α and β-B subunit mRNA vary

cyclically at different stages, the highest levels of both α and β-B subunit expression were observed in stages XIII-I and lowest in stages VII-VIII. These changes could be due to a change in Sertoli cell number per stage, or to a change in subunit expression per Sertoli cell. As the former does not vary significantly (Wing & Christensen, 1982) these results suggest that inhibin mRNA (and therefore probably inhibin) are produced stage-dependently (Bhasin *et al*, 1989). The physiological relevance of this in the post-pubertal rat remains uncertain (see later) however in these studies a paracrine role for inhibin has been postulated: Since FSH is maximally bound in stages I and II whereas minimal binding occurs in stages VII-VIII it is possible that, during these stages FSH stimulates inhibin secretion.

Recent advances in the purification and culture of Sertoli cells and germ cells have made it possible to study the role of certain types of germ cells in stimulating many Sertoli cell secretions. It now seems possible that the stimulus for secretion of a number of Sertoli cell proteins is not due to an inherent cyclicity of the Sertoli cell but the result of selective germ cell classes (for example pachytene spermatocytes and round spermatids) producing paracrine factors which modulate the production of a number of Sertoli cell secretions (Jegou *et al*, 1988). These will be discussed later in the context of Sertoli cell-germ cell interactions.

1.1.4. Paracrine interactions.

For any organ or tissue consisting of numerous functionally different cell types the existence of local communication to co-ordinate their function is necessary for the tissue to function as a unit. Whilst the gonadotrophins LH and FSH are secreted by the pituitary and are the means by which the brain exerts overall control of testicular function and fertility (discussed later), several morphological and biochemical observations suggest strongly that local interactions are absolutely essential for the efficient running of spermatogenesis. Such interactions are mediated by the release of soluble factors (mainly proteins) and occur between various cell types within the testis including Sertoli cells, germ cells, peritubular cells, Leydig cells and vascular endothelial cells. Inter-tubule paracrine interactions (i.e. those between the tubule and the interstitium and those within the interstitium are discussed later).

1.1.4.1. Sertoli cell-germ cell interactions.

The existence of paracrine factors released by both Sertoli and germ cells for communication and co-ordination of complex events within the seminiferous tubule have been suspected for many years (Sharpe, 1984). Throughout spermatogenesis there are numerous mitotic and meiotic divisions of germ cells, resulting in the differentiation of stem cells into highly specialised spermatozoa. These events must be timed precisely.

Indirect evidence for cellular communication between Sertoli and germ cells has come from investigations using electron microscopy. These have demonstrated that Sertoli cells have gap junctions with their germ cells (McGinley et al, 1979). Additionally, complex ultrastructural changes which occur in the region of the Sertoli cell close to the germ cells during spermatogenesis strongly suggest the Sertoli cells secrete compounds directly necessary for germ cell survival (Russell, 1980). Two Sertoli cell secretions which are obligatory for germ cell development are lactate and pyruvate which act as an energy source for the developing germ cells (Jutte et al, 1983). Because of the structural relationship between germ cells and Sertoli cells it is probable that germ cell requirements are met exclusively by the Sertoli cell. In addition, due to the fact that each individual stage of spermatogenesis always contains the same germ cell profile, it is also probable that particular proteins are synthesised and secreted stage-dependently for germ cell utilisation, possibly stimulated by particular germ cell types per stage or via the transport of these substances from blood and interstitial fluid across the Sertoli cell cytoplasm.

More direct evidence has demonstrated (*in vivo* and *in vitro*) that when germ cells are destroyed a decrease in Sertoli cell specific functions occurs (Jegou et al, 1984; Fritz & Tung, 1987). Variations in Sertoli cell secretions have been identified *in vitro* following the addition of sub-classes of germ cells including pachytene spermatocytes, round spermatids and residual bodies to Sertoli cell monocultures. In Sertoli cell monocultures FSH stimulates ABP production by Sertoli cells, which is enhanced by co-culture with pachytene spermatocytes (Le Magueresse et al, 1986; Le Magueresse & Jegou, 1986), but not by co-culture with round spermatids (Galdieri et al, 1984). Although the mechanism for this enhancement is unknown, germ cells and Sertoli cells form gap junctions *in vitro* (Ziparo et al, 1982; Galdieri et al, 1984), as they do *in vivo*. However, other workers have demonstrated that media conditioned by short-term (24 hour) culture of isolated pachytene spermatocytes when added to Sertoli cells *in vitro* stimulate ABP production and decreased FSH-induced oestradiol production (Le Magueresse & Jegou, 1986; 1988). Thereby

suggesting that a soluble paracrine mediator was transferred from germ cells to Sertoli cells. Further studies have demonstrated that transferrin secretion by isolated Sertoli cells is enhanced in the presence of pachytene spermatocytes, early spermatids and liver epithelial cells (Le Magueresse *et al*, 1988). Furthermore spent media from pachytene spermatocytes and early spermatids (but not liver epithelial cells) also stimulated transferrin production. Therefore germ cells may be capable of controlling their own supply of iron via influences upon transferrin secretion by the Sertoli cell. In addition, germ cells increase phosphorylation of two specific Sertoli cell proteins (Ireland & Welsh, 1987). The identity of these proteins has yet to be determined. Recent data has also shown that the secretion of inhibin by Sertoli cells in culture is enhanced by co-culture with early spermatids (Pineau *et al*, 1990). This finding is an important one as it suggests that germ cells can modulate the synthesis and secretion of Sertoli cell proteins and can communicate, (all be it indirectly) with the pituitary, via stimulating or inhibiting the release of inhibin and hence FSH (see later).

There is also evidence to suggest that whilst certain sub-classes of germ cells may control Sertoli cells during the development of spermatogenesis, other classes take over this control following the complete establishment of spermatogenesis. Whilst pachytene spermatocytes strongly influence ABP secretion when added to cultures of immature rat Sertoli cells (Jegou *et al*, 1986; Le Magueresse & Jegou, 1986; 1988), there is a correlation *in vivo* between the number of late spermatids and ABP levels following maturation (Jegou *et al*, 1984; 1986; Pinon-Lataillade *et al*, 1988). These experiments have been performed using X-irradiated animals to deplete spermatogonia. In addition, a rapid decline in the secretion of a number of Sertoli cell products including seminiferous tubule fluid, ABP and inhibin following partial (Jegou *et al.*, 1984) or major depletion of germ cells *in vivo* has been observed after irradiation (Au *et al*, 1983; Jegou *et al*, 1983).

Further evidence in support of this but demonstrated *in vivo* shows: a) The production of ABP and seminiferous tubule fluid by the testis of immature (25 to 35 day old) rats depleted of spermatocytes and early spermatids using perinatal irradiation or cryptorchidism at 14 to 17 days of age is comparable to that of 15 to 20 day old animals (Jegou *et al*, 1983; Karpe *et al*, 1981; Rich, 1979). b) Oestrogen secretion from isolated rat Sertoli cells following perinatal irradiation to create Sertoli cell only tubules is similar to oestradiol secretion by 7 to 10 day old control rats (Rommerts *et al*, 1982).

These data re-inforce the belief that Sertoli cell-germ cell interactions do exist during the development and maintenance of spermatogenesis *in vivo*. To identify these factors and elucidate how Sertoli cell function is regulated by them and, inturn, how Sertoli cell derived factors mediate germ cell function is critical for understanding the control mechanisms in spermatogenesis. In addition to Sertoli cell - germ cell interactions, other Sertoli cell - testicular cell interactions exist.

1.1.4.2. Sertoli cell-peritubular cell interactions.

Surrounding each seminiferous tubule is a layer of peritubular myoid cells which lie external to the basement membrane (Ross & Long, 1966). A basal lamina is present between the peritubular cells and the Sertoli cells within the seminiferous epithelium. It is composed of collagen fibres and extracellular matrix components deposited in 3 to 5 alternating layers, separated by thin layers of microfibrils and devoid of blood vessels or nerves (de Krester *et al*, 1975).

Recently, evidence obtained *in vitro* has established that peritubular cells play a dynamic role in the local co-ordination of testicular function. Due to the proximity of Sertoli cells and peritubular cells the latter are able to influence the former both morphologically and hormone dependently.

When cultured separately, Sertoli cells and peritubular cells secrete different extracellular matrix components which together form the basal lamina, but neither cell is capable of synthesising all components alone (Skinner *et al*, 1985). Morphological examination of Sertoli cell - peritubular cell co-cultures has shown that formation of this extracellular limiting membrane only takes place in co-culture (Tung & Fritz, 1980). Using enzyme linked immunosorbent assays and immunocytochemical techniques evidence has been obtained to suggest that whilst peritubular cells secrete fibronectin alone, Sertoli cells secrete laminin and both cell types release type IV collagen (Tung & Fritz, 1984; Tung *et al*, 1984). All are necessary for formation of the basal lamina.

The role of this basal lamina is unclear but it is thought to promote histotypic expresssion of Sertoli cells *in vitro* resulting in columnar rather that squamous monolayer cultures, with adjacent cytoplasmic processes and junctional complexes between adjacent cells. These cultures are more representative of their *in vivo* counterparts (Tung & Fritz, 1984; Byers *et al*, 1986).

Peritubular cells maintained in mono-culture require serum for survival, but in co-culture with Sertoli cells they can survive in serum-free medium for an extended period and stimulate Sertoli cell activities for a

prolonged period. For example, in co-culture there is an increase in the secretion of ABP and transferrin by Sertoli cells in comparison to Sertoli cell cultures alone (Tung & Fritz, 1980; Hutson & Stocco, 1981; Holmes *et al*, 1984).

The *in vitro* evidence presented so far indicates that cell-cell contact is required for an interactions to take place. Recent evidence suggests that soluble paracrine factors may play a role in communication between Sertoli cells and peritubular cells. Skinner and Fritz (1985) demonstrated that peritubular cells produce a non-mitogenic factor of 70 kDa (Skinner & Fritz, 1986). This factor, named P-Mod-S, stimulated transferrin, ABP and cAMP production by Sertoli cells *in vitro* to the same extent as did maximal hormonal stimulation. What stimulates the production of P-Mod-S by peritubular cells ? Gonadotrophins do not bind to isolated peritubular cells (Steinberger *et al*, 1975; Fritz *et al*, 1975) but androgen receptors are present in peritubular cells (Sar *et al*, 1975). Furthermore, the production of P-Mod-S is stimulated by testosterone (Skinner & Fritz, 1985). These observations are supported by indirect experimental evidence obtained *in vivo* and *in vitro*. *In vivo*, Bressler and Ross (1972) observed peritubular cell differentiation in testosterone-treated hypophysectomised adult rats given intratesticular implants of immature testicular tissue. *In vitro*, Hovatta (1972) noted that the anti-androgen cyproterone acetate prevented the development of rat peritubular cells . Further work by Hutson *et al* (1987) suggests that P-Mod-S and FSH act through different mechanisms to stimulate ABP and cAMP secretion from Sertoli cells *in vitro*, and that these effects on Sertoli cells are exerted at the level of translation by increasing the rate of peptide elongation (Hutson *et al*, 1987).

These results have led to the conclusion that androgens act at two levels on Sertoli cells, viz a direct action via Sertoli cell androgen receptors and an indirect action mediated via peritubular cell androgen receptors and P-Mod-S.

Due to the potential importance of P-Mod-S in spermatogenesis Skinner *et al* (1988) have identified two forms, named A and B, which differ in apparent molecular weight of 54 to 56 kDa and 59 kDa respectively. Radiolabelled experiments have demonstrated the active synthesis and secretion of P-Mod-S by peritubular cells in culture. The relationship between P-Mod-S A and B is unknown, however these purified forms were demonstrated to have greatest effects on ABP and transferrin secretion by Sertoli cells in culture when compared to other regulatory agents including FSH, insulin, retinol and testosterone. The physiological significance of P-Mod-S as a possible paracrine regulator of testicular function has yet to be elucidated.

1.1.5. Techniques employed to study Sertoli cells *in vitro*.

The development of techniques for the isolation and culture of testicular cells has, for a number of years, been recognised as a valuable means of investigating male reproductive function. The culturing of individual cell populations at various stages of their development has allowed a fine dissection of the fundamental activities of each. Due to the heterogeneous cell population of the testis and the complexity of spermatogenesis this would not have been possible *in vivo*. The proposed role in spermatogenesis of a number of testicular cells and their secretions have been established *in vitro*. In particular, Sertoli cell culture techniques have been important in demonstrating the probable roles of the Sertoli cell, the origin of its secretory components, their stage dependent cyclical production (Parvinen, 1982) and the involvement of germ cells, Leydig cells and peritubular cells in controlling their production (Galdieri *et al*, 1984; Le Magueresse *et al*, 1988; Le Magueresse & Jegou, 1986; 1988; Skinner & Fritz, 1986; Ireland & Welsh, 1987). These have been discovered over the past decade using isolated tubule cultures and cell preparations.

1.1.5.1. Primary cultures.

Sertoli cells were first prepared from the testes of 20 day old rats by several different groups (Dorrington *et al*, 1975; Steinberger *et al*, 1975; Welsh & Weiber, 1975). The cultured cells form a non-dividing monolayer consisting of approximately 90% Sertoli cells (Fritz *et al*, 1975). Since these initial studies, innovations in culture technique have meant that germ cell and peritubular cell contamination can be reduced at the isolation stage by repetitive washing and sedimentation (Mather & Philips, 1984), by filtration (Steinberger *et al*, 1975) or by brief exposure to hypotonic Tris buffer after plating (Galdieri *et al*, 1981; Wagle *et al*, 1986). The inclusion of a hyaluronidase enzyme digestion step during culture generation removes 99% of peritubular cells (Tung *et al*, 1984). As these contaminants in pure Sertoli cell preparations can influence Sertoli cell function (Galdieri *et al*, 1984; Skinner & Fritz, 1986) their numbers should be established in culture. Markers used to identify peritubular cells include desmin (Palomli *et al*, 1988) known to be a component in intermediate filaments in these cells (Vitanen *et al*, 1986) and alkaline phosphatase, a cell surface enzyme (Chapin *et al*, 1987).

A major advantage of any cell culture system is the ability to study an individual cell type in the absence of others. With a process as complicated as spermatogenesis with numerous interactions taking place simultaneously the isolation of a cell type such as the Sertoli cell could be considered

unphysiological, since many of its functions rely on paracrine interactions with other cells. In order to study these interactions a number of protocols have been adopted: 1) To couple individual cell populations forming co-cultures. Recent advances in cell isolation have made it possible to purify peritubular cells (Skinner & Fritz, 1984) and specific sub-classes of germ cells (Romrell *et al*, 1976; Meistrich, 1977; Meistrich *et al*, 1981; Bucci *et al*, 1986), the latter using velocity sedimentation and centrifugal elutriation. These specific germ cell enriched preparations can be added back to Sertoli cells in culture to observe the influence of germ cells on Sertoli cells (Le Magueresse, 1986; Le Magueresse & Jegou 1986; 1988). 2) To examine the effect of medium conditioned by one cell type on the functions of another separate cell preparation. This technique has been used to study intra-tubular and intratesticular paracrine communications between Sertoli cells and other testicular cells.

Whilst Sertoli cells cultured in serum free medium are responsive to hormones and secrete proteins into the medium for 1 to 2 weeks after plating their responsiveness begins to decline after 6 to 8 days (Karl & Griswold, 1980; Skinner & Griswold, 1982). Sertoli cells cultured in the presence of germ cells or peritubular cells show a more sustained responsiveness probably because under co-culture conditions a more physiological environment is formed (Skinner & Fritz, 1985).

A further advance has been the use of extracellular matrix in Sertoli cell cultures (Tung & Fritz, 1984). In the absence of an extracellular matrix, Sertoli cells become squamous in appearance and lose a number of structure-function relationships with germ cells. When Sertoli cells are cultured on extracellular matrix they retain their columnar shape and are morphologically more representative of their *in vivo* counterparts. This technique has been enhanced by the introduction of filters impregnated with extracellular matrix - known as bicameral chambers (Byers *et al*, 1986) which allow the polarity of Sertoli cell secretion to be studied *in vitro*. This technique has been used to investigate the bidirectional secretion of a number of Sertoli cell proteins including ABP (Hadley *et al*, 1987), transferrin (Janecki *et al*, 1988), plaminogen activator (Ailenburg & Fritz, 1988) and inhibin (Handelsmen *et al*, 1989).

The refinements outlined above have made it possible to culture Sertoli cells alone, in the presence of germ cells or selectively enriched preparations of germ cells and in the absence or presence of peritubular cells or an extracellular matrix. However, there are also a number of disadvantages to Sertoli cell cultures particularly since these cells are highly differentiated and

play a complex role in spermatogenesis. A key question is how representative are cultured Sertoli cells in comparison to their *in vivo* counterparts, and perhaps more importantly what relevance do Sertoli cell cultures generated from immature animals have to the function of Sertoli cells in the adult testis?

Primary cultures are used extensively to investigate Sertoli cell function *in vitro* . These cells retain many of the differentiated characteristics of their *in vivo* counterparts, however, they are inherently variable and only have a limited lifespan. An alternative is cell lines which are more uniform but do not retain all of these characteristics and therefore are not truly representative of Sertoli cells (Mather *et al*, 1982).

Whilst it is possible to culture Sertoli cells isolated from 28 day old immature rat testes (Gray & Beamond, 1984) and up to 35 days old, it is extremely difficult to isolate and culture Sertoli cells from adult rat testes (Steinberger *et al*, 1975). The reasons for this are three fold: 1) As maturation progresses, the generation of particular classes of germ cells occurs and therefore elongate spermatids develop at around 35 days of age (Russell *et al*, 1987). These cells contain an acrosome which, during the enzymatic isolation procedure is activated causing the agglutination of cells in the digest. In addition, the release of DNA from lysed cells potentiates this problem. Although the latter problem can be resolved with the addition of DNAase to the digest it is still difficult to achieve a comprehensive enzymatic digestion. 2) Sertoli cells which are isolated successfully from adult rat testes do not adhere to polystyrene or extracellular matrix coated polystyrene culture dishes. A possible reason for this could be that adult Sertoli cells are generally less responsive in comparison to their immature counterparts and as such any secretory factors which aid in the adhesive process are not produced. By contrast Leydig cells can be isolated and purified from mature rat (and human) testes using continuous or discontinuous density gradients made from Percol (Simpson *et al*, 1987).

The culturing of Sertoli cells from younger animals is feasible but not well defined. Sertoli cells generated from rats of 15 days old or less are complicated by the vigorous mitotic activity of contaminating peritubular cells which are difficult to remove with hyaluronidase. This proliferation can be prevented by the inclusion of cytosine arabinaside in the medium (Tung *et al*, 1984) however this also prevents the mitotic activity of Sertoli cells in culture. In addition other structural changes occurring at this time including the formation of the blood-testis barrier (Setchell, 1969) and the absence of many complements of germ cells. Therefore these cells are probably far less

representative of the situation in the adult testis than Sertoli cells derived from 28 day old rats.

Through cytoplasmic connections Sertoli cells are in continual contact with germ cells and adjacent Sertoli cells. During the isolation procedure these connections are broken and Sertoli cells lose their structural cellular associations. In culture, aggregates of Sertoli cells and germ cells settle to eventually form confluent monolayers of Sertoli cells and germ cells. Adjacent Sertoli cells in culture may therefore be derived from different stages of spermatogenesis *in vivo*, and, as such, cyclically secrete different proteins. Thus, a non-uniform population of Sertoli cells is formed, each releasing specific substances governed by its complement of germ cells, although this is educated conjecture at present. This problem can be overcome by removing all germ cells, thus establishing a uniform Sertoli cell population, and replacing them with an enriched preparation of different sub-classes of germ cells which adhere to Sertoli cells. This technique has been adopted for some germ cell classes but it is impossible to accurately isolate enriched populations of germ cells other than pachytene spermatocytes and round spermatids. For these two cell types, Meistrich *et al* (1981) have recovered fractions as pure as 98% and 93% respectively but attempts to purify type A and B spermatogonia to this purity have so far been unsuccessful (Bucci *et al*, 1986).

Bicameral chambers were an innovation of normal culture techniques to develop "psuedo normal conditions" with different ionic concentrations on either side of the chamber, similar to those which exist *in vivo*. They have since been adopted as a technique for studying the bidirectional secretion of Sertoli cells *in vitro*. This experimental model however has a number of pitfalls. Janecki and Steinberger (1988), using ABP and transferrin as indices of Sertoli cell secretory function have evaluated this model. They identified differential binding of both proteins to the filter which separates the upper and lower chambers such that reliable evaluation of vectorial protein secretion was only obtained by pre-treating the filter with albumin to reduce this non-specific binding. In general however, it remains to be proven how physiological and representative bicameral chambers are. It is generally presumed that because Sertoli cells are more like their *in vivo* counterparts, (in terms of shape), that their functions are comparable to *in vivo*. This is almost certainly an over simplification and it is probable that while the secretion of some Sertoli cell products may well be enhanced and more representative of the *in vivo* situation others will not.

1.1.5.2. Seminiferous tubule cultures.

A substantially more physiological approach was adopted by Hagernas (1978). The technique involved the mechanical dissection and culturing of lengths of seminiferous tubules. The obvious advantage of this system is that the integrity of the seminiferous epithelium is retained (barring mechanical damage) whilst interstitial tissue is removed without the problems associated with enzymic digestion.

Parvinen (1982) refined the technique by illuminating the tubule from below to identify, dissect and culture specific stages of the spermatogenic cycle. Using this technique Parvinen noted numerous cyclical changes in Sertoli cell products which correlated to specific stages of the spermatogenic cycle. The advantage of this technique is that specific stage(s) can be grouped and cultured separately or together.

The major problem to date with seminiferous tubule cultures is that it is unknown what degree of normal morphology is maintained within the epithelium and for how long in culture. The disadvantage associated with culturing specific lengths of seminiferous tubules in individual stages is that some stages are extremely small and their duration is short (e.g. stage III lasts 6h). Therefore a stage of short duration will probably progress into the next stage *in vitro* before adequate measurements have been made. Evidence for the degree of normal morphology post-culture could probably be obtained from histological preparation and microscopic examination of these cultured tubules, although the very size and fragility of these tubules would make it difficult to prepare them for examination without inducing histological artifact.

1.1.5.3 Whole organ culture.

Whole organ culture represents another method for the study of testicular physiology. The technique is complex, difficult, has a short lifespan and produces a very limited amount of information. Lee and Nagayama (1980) have used isolated perfused testis to study the metabolism of benzo(a)pyrenes, maintaining the testis relatively physiologically for several hours. The technique allows the investigation of possible metabolic pathways for the degradation of possible testicular toxicants in the whole organ but offers little advantage over other techniques. It has the additional disadvantage in that the testis is considered to be on the brink of hypoxia and, as such, perfusion fluids require a high oxygen tension to prevent oedema, hypoxia and cellular degeneration.

1.1.6. Leydig cells.

Franz Leydig in 1850 first described the characteristic Leydig cells of the interstitium in several species of mammals. They are highly differentiated cells which specialise in the synthesis and secretion of androgens, primarily testosterone, necessary for the maintenance of spermatogenesis but with a host of effects on non-reproductive tissues (bone, muscle, skin etc. - Sharpe, 1988). Synthesis and secretion of androgens is stimulated by leutinising hormone (LH) secreted by the pituitary. In the rat, Leydig cells have a prominent nucleus and lie in close association with the vasculature of the tubule (Fawcett *et al*, 1973; Christensen, 1975; Kerr & de Kretser, 1981). Morphometric analysis has shown that there are 22×10^6 Leydig cells per gram of testis in the rat (Mori & Christensen, 1980) comprising approximately 3% of the total testicular tissue volume.

A discussion of the pituitary testicular axis with respect to the Leydig cells and testosterone production is given in section 1.2, however a number of paracrine regulators of Leydig cells and testosterone production have also been postulated and are discussed in this section, although with respect to steroidogenesis, it must be remembered that the major regulator of this process is LH and paracrine factors only modulate this process.

1.1.6.1. Sertoli cell and Leydig cell paracrine interactions.

The first factor to be recognised was leutinising hormone releasing hormone (LHRH) like factor secreted by the Sertoli cells (Sharpe *et al*, 1981). Although this factor is not proven to be LHRH, it is now clear that Leydig cells have specific receptors for LHRH. Both LHRH and its agonist analogues can affect Leydig cell steroidogenesis in an inhibitory or stimulatory manner depending upon the length of exposure, the former occurring only after a prolonged exposure (3 days or more), and the latter during a short exposure time (up to 24 hours) (Browning *et al*, 1983; Sharpe, 1986). In addition, LHRH can alter testicular blood flow and IF volume, both of which provide further ways of regulating the concentration of testosterone in testicular interstitial fluid (Sharpe, 1986).

Several investigators have described the presence of a factor from Sertoli cell spent media distinct from the LHRH-like factor, which increases testosterone secretion by Leydig cells *in vitro* (Grotjan & Heindel, 1982; Janecki *et al*, 1985; Papandopoulos *et al*, 1987; Verhoeven & Cailleau, 1987). These factor(s) modulate LH action on Leydig cells and are thermolabile, FSH-dependent, have molecular weights between 10 kDa and 50 kDa

A similar factor has been documented to be present *in vivo* in IF from normal rats (Sharpe & Cooper, 1984) and the levels of this factor can vary depending on the intratesticular testosterone level (Sharpe, 1986). Conversely, a seminiferous tubule factor of probable Sertoli cell origin that inhibits LH-stimulated testosterone secretion by Leydig cells *in vitro* has been described (Syed *et al*, 1986; 1987). It has been suggested that differential secretion of this inhibiting factor may be an important regulator of varying testosterone concentrations during the different ages of the male rat (Syed *et al*, 1987).

A Sertoli cell factor that increases Leydig cell aromatase activity in the mature rat has also been described recently (Carreau *et al*, 1988). This thermolabile, FSH-independent factor appears to differ from the Sertoli cell factor which regulates testosterone because the aromatase regulating factor requires protein synthesis for its action whilst the testosterone regulating factor does not. There are also reports of an aromatase inhibiting factor produced stage-dependently at stages VII-VIII, the most androgen dependent stage of the cycle (Boitani *et al*, 1981). The presence of this factor suggests that Sertoli cells are able to maintain high intratesticular concentrations of testosterone by ensuring minimal conversion of testosterone to oestrogen by the neighbouring Leydig cells.

A number of studies have also reported the presence of the renin-angiotensin system in Leydig cells. Leydig cells have specific angiotensin II receptors and angiotensin II binding activates the subunit of adenylate cyclase, thus inhibiting gonadotrophin stimulation of cAMP, resulting in decreased testosterone production (Khanun & Dufau, 1988). The significance of this is unknown.

Numerous investigators have studied the role of pro-opiomelanocortin (POMC) derived proteins in the testis. Among these, β-endorphin was postulated as a paracrine modulator of Sertoi cell function, secreted by Leydig cells (Bardin *et al*, 1987). Studies strongly suggest that POMC-derived peptides are synthesised mainly in the testicular interstitium and in most studies, the Leydig cell has been considered to be the predominant site of POMC synthesis (Tsong *et al*, 1982; Chen *et al*, 1984). Li *et al* (1989) tested this hypothesis using ethane dimethane sulphonate (EDS), the Leydig cell cytotoxin. They demonstrated that testicular levels of POMC-derived peptides and POMC-like mRNA were not significantly altered *in vivo* after EDS, despite the complete absence of Leydig cells. Indeed, the demonstration of a POMC mRNA transcript in a crude interstitial preparation would suggest that POMC-derived peptides may be present in interstitial cells other than Leydig cells,

probably macrophages. POMC-like mRNA has been identified in spleen macrophages (Lolait *et al*, 1986). which would add further evidence to POMC-derived proteins being secreted from macrophages rather than Leydig cells.

Leydig cells also have receptors for arginine vasopressin (AVP) (Meidan & Hsueh, 1985), and AVP can modulate Leydig cell steroidogenesis (Sharpe & Cooper, 1987). Catecholamines may also affect intratesticular levels of testosterone (Damber & Janson, 1978) whilst corticotrophin-releasing factor also has effects on Leydig cells *in vitro* (Yoon *et al*, 1988) as does interferon (Orava, 1988) and atrial naturetic factor (Foresta *et al*, 1987). Insulin can also regulate Leydig cell steroidogenesis and Leydig cells have receptors for insulin and insulin-like growth factor I (IGFI) (Lin *et al*, 1986). Transforming growth factor β (TGFβ) has also been shown to decrease testosterone synthesis in cultured adult rat Leydig cells *in vitro*, whilst TGFα had no effect (Lin *et al*, 1987).

Further paracrine interactions between Leydig cells and peritubular cells and possibly germ cells have also been postulated. Oxytocin is a secretory product of Leydig cells which is thought to act on the myoid cells of the tubules to bring about their contraction. These contractions might aid in the transport of the shed sperm to the rete testis (Sharpe, 1986). It may also act in an autocrine manner on the Leydig cell, since there is some indication that Leydig cells may have oxytocin receptors (Tahri-Joutei & Pointis, 1988).

In summary, there is a complex inter-relationship among the various components of the testis, which are just beginning to be understood. It appears that through paracrine, endocrine and possibly autocrine mechanisms, the function of each cell type is modulated to integrate its activity with that of the other cell types. However, without endocrine support, on which the whole system depends, spermatogenesis would break down. The hormonal regulation of spermatogenesis is discussed in the next section.

1.2. HORMONAL CONTROL OF SPERMATOGENESIS.

The initiation, development and maintenance of spermatogenesis are under the dual control of the endocrine hormones; follicle stimulating hormone (FSH) and leutinising hormone (LH). The brain exerts overall control over gonadal function via the release of these two gonadotrophins from the pituitary as well as prolactin, although the role of the latter is rather unclear. In addition to the gonadotrophins, paracrine factors exist to co-ordinate functions of testicular cells at the cellular level (discussed earlier).

FSH and LH are complex glycoproteins consisting of two dissimilar sub-units termed α and β. The α-subunit is common to FSH, LH, human Chorionic Gonadotrophin (hCG) and thyroid stimulating hormone (TSH) while the β subunit confers specificity for biological activity (Pierce & Parsins, 1981). The association of the α and β subunits results in a biologically active dimer (reviewed by Chin *et al*, 1985). Both FSH and LH are released from the anterior pituitary into the circulation under the drive of leutinising hormone releasing hormone (LHRH) released in a pulsatile manner from the hypothalamus (Negro-Vilar & Valenca, 1988).

FSH and LH act specifically on Sertoli cells and Leydig cells respectively. In response to FSH, Sertoli cells secrete inhibin while Leydig cells secrete testosterone in response to LH stimulation. In a classical negative feedback manner, testosterone regulates the secretion of both LH and FSH (particularly the former) while inhibin preferentially regulates FSH, although it may also regulate LH secretion to a degree. Matters have however been complicated by the discovery that Leydig cells secrete inhibin (Risbridger *et al*, 1989; Maddocks & Sharpe, 1989).

1.2.1. Control of FSH secretion.

The role of inhibin and testosterone in the control of FSH secretion is still unclear. Early arguments against the existence of inhibin were based on results which demonstrated that the adult male rat testosterone inhibited FSH and LH secretion in a dose-dependent manner (Lee *et al*, 1972; Baker *et al*, 1976; Decker *et al*, 1981; de Kretser *et al*, 1987). However in castrate adult males very high doses of testosterone could totally suppress LH levels but did not lower FSH levels to less than the normal range (Decker *et al*, 1981; de Kretser *et al*, 1987). Further experiments performed *in vivo* utilising the Leydig cell cytotoxin EDS have demonstrated that rising FSH and LH levels are accompanied by falling testosterone levels, suggesting that testosterone not only keeps LH but, also FSH in check (Jackson *et al*, 1986). In these experiments however, FSH levels were elevated to only 50% of the levels detected in castrate animals, indicating the existence of an intratesticular factor which inhibits further rises in FSH. Furthermore, in bilaterally cryptorchid rats the rise in serum FSH levels to 50% of the levels in castrate animals has been attributed to diminished inhibin secretion as serum testosterone remains at normal levels (Au *et al*, 1983). The concept of a dual role of inhibin and testosterone in the control of FSH secretion was further supported in cryptorchid rats administered EDS. EDS administration to such animals results in a further rise of FSH levels into the castrate range

(O'Leary *et al*, 1986). In parallel with the development of a new generation of Leydig cells, testosterone levels were restored and FSH levels fell to the pre-treatment (cryptorchid) level. These data show that in the rat under a number of experimental conditions the control of highly elevated FSH levels can be attributed to both testosterone and inhibin.

Studies have also utilised crude preparations of body fluids (follicular fluid [FF], testicular extracts etc. - known to contain inhibin activity), to examine the effects of inhibin in vivo in rabbits (Laborde *et al*, 1984) and rats (Lorenzen *et al*, 1981; Lipner & Dhanarajan, 1984). Testosterone and inhibin have been demonstrated to be most effective in suppressing FSH *in vivo* when administered together. Low doses of testosterone replacement in castrate rats was initially effective in suppressing gonadotrophins. However after 6 days FSH suppression was incomplete but was restored by the co-administration of porcine FF (Summerville & Schwartz, 1981). In another study examining acutely castrated male rats, treatment with testosterone produced dose-dependent suppression of FSH and LH whilst FF suppressed FSH alone (Jones *et al*, 1985). In this study the elevation of FSH in chronically castrate male rats was not suppressed by testosterone alone whilst porcine FF was effective. Studies utilising immunoneutralisation techniques have also been instrumental in demonstrating the role of testosterone and inhibin in FSH control. Passive immunisation of rats against testosterone secretion has provided further evidence that although testosterone had a significant inhibitory effect on FSH secretion, inhibin had a major role to play in the regulation of FSH secretion (Main *et al*, 1980).

Although the experiments described above have examined the role of testosterone and Inhibin in FSH regulation in animal models (rat and rabbits), their relationship to normal physiological conditions in the adult male rat remains unclear. Recent data has demonstrated that during maturation in the rat levels of serum inhibin decline progessively through puberty and into adulthood (Rivier *et al*, 1988; Culler & Negro-Vilar, 1988; Maddocks & Sharpe, 1990). During this period a direct correlation between declining serum FSH and serum inhibin levels exists. Therefore in the normal adult rat, what contribution does inhibin make in controlling FSH secretion? Rivier *et al* (1988) and Culler & Negro-Vilar (1988) have shown that immunoneutralisation of inhibin in rats increases plasma FSH levels but **only** at 10-24 days of age and not in older animals. These results suggest that inhibin plays a physiological role in suppressing FSH secretion in infantile (pre-pubertal) rats whilst testosterone is the over-riding factor in controlling FSH output in the adult rat (Culler & Negro-Vilar, 1988)

In conclusion, whilst the control of FSH secretion is affected by both testosterone and inhibin, it seems likely that inhibin negatively regulates FSH output only in pre-pubertal rats whilst testosterone takes over this regulation through puberty and into adulthood. However it apears that both inhibin and testosterone can work through independent mechanisms in the regulation of FSH secretion (McLachlan *et al*, 1988; de Kretser & Robertson, 1989). This leaves us with the question: What is the role of inhibin in the normal adult male? Several pieces of evidence have suggested that inhibin and/or inhibin related peptides may have a paracrine role to play in spermatogenesis (Lee *et al*, 1988; van Dissel-Emiliani *et al*, 1989; Bhasin *et al*, 1989) (discussed earlier).

1.2.2. Role of FSH.

The importance of FSH in the initiation and expansion of spermatogenesis in mammals during puberty is well documented (Russell *et al*, 1987), however its role in the normal adult male is poorly understood (Sharpe, 1989). FSH receptors are present on Sertoli cells, which constitute the major site of FSH bin ding in the testis (Means & Vaitukaitis, 1972; Bhalla *et al*, 1974; Steinberger & Chowdhury, 1974). Although FSH is known to stimulate a number of diverse functions of the Sertoli cell including energy metabolism, protein synthesis, cell shape changes and cell division, its role in the adult animal remains a mystery.

Passive immunisation of adult rats with FSH antibodies has relatively little or no effect on spermatogenesis (Davies *et al*, 1979; Dym *et al*, 1979). Furthermore qualitatively normal spermatogenesis can be maintained in hypophysectomised adult rats by testosterone alone (Ahmad *et al*, 1979; Buhl *et al*, 1982), however testicular weight (and therefore sperm output) is subnormal in these studies (Sharpe, 1987). It is now well established that quantitatively normal spermatogenesis can be maintained in rats administered long acting testosterone esters in the absence of Leydig cells for at least 10 weeks despite the fact that serum FSH levels are low or undetectable (Sharpe *et al*, 1988). Perhaps FSH is not required to maintain spermatogenesis in the adult rat? The overall picture however is not so simple. Bartlett and co-workers (1989) have demonstrated that treatment of hypophysectomised adult rats with highly purified FSH plus a low dose of testosterone for 2 weeks was able to maintain spermatogenesis far more effectively than testosterone administration alone, and without altering the intratesticular level of androgens.

Based on these results, the current thinking is that FSH facilitates the actions of testosterone on spermatogenesis but that qualitatively normal

spermatogenesis can be maintained in the absence of FSH in both the rat and man (reviewed by Sharpe, 1989). As FSH has been shown to increase the number of androgen receptors in cultured immature rat Sertoli cells (Themmen *et al*, 1989), the likely basis for the facilitatory effect of FSH on androgen action in the adult testis is by increasing androgen receptor levels. This would effectively increase sensitivity of the Sertoli cell to testosterone with the result that quantitatively normal spermatogenesis could be maintained with lower levels of testosterone than would be possible in the absence of FSH. As the FSH-responsive stages (I-V) of the spermatogenic cycle in the rat precede the androgen repsonsive stage (VII) it is likely that this facilitatory effect of FSH is a normal physiological phenomenon.

FSH is known to stimulate a number of Sertoli cell secretions including ABP (Vernon *et al*, 1974; Steinberger *et al*, 1975; Hansson *et al*, 1976), several secretory proteins with unknown functions (Wilson & Griswold, 1979; Cheng *et al*, 1986), energy metabolism (Jutte *et al*, 1981; 1983), plasminogen activator (Lacroix *et al*, 1977; Lacroix & Fritz, 1982; Fritz & Karmally, 1983) and the iron-carrier protein transferrin (Perez-Infante & Mather, 1982; Skinner & Griswold, 1982; Holmes *et al*, 1984). Although a role in spermatogenesis for a number of the above Sertoli cell secretions has been postulated, their necessity in the process of spermatogenesis is unknown.

A further way in which FSH might support spermatogenesis in the adult is via a secondary effect on the Leydig cell. Evidence obtained from both rodents and man suggest that FSH can stimulate the production of one or more paracrine factors by the Sertoli cell which can act on the Leydig cell to enhance testosterone production in respone to LH (Verhoeven & Cailleau, 1987), FSH may also be involved in controlling Leydig cell numbers (Kerr & Sharpe, 1985). FSH significantly increases testosterone secretion in perfused rabbit testis, and FSH treatment of immature hypophysectomised rats induces Leydig cell hypertrophy, increases LH receptor number and testosterone secretion *in vivo* and *in vitro* (van Beurden *et al*, 1976; Chen *et al*, 1977; Benahmed *et al*, 1981; Moger & Murphy, 1982; Kerr & Sharpe, 1985). This effect of FSH is only evident when Sertoli cells are present, and appears to be specific for FSH alone since LH alone does not produce the same effect (Benahmed *et al*, 1985; Kerr & Sharpe, 1985).

In summary, although FSH is essential for the initiation of spermatogenesis, its maintenance in the adult is dependent on testosterone (Sharpe *et al*, 1990). FSH induces changes in Sertoli cell morphology and increases the secretion of a number of factors in culture which are thought to be

required for spermatogenesis. However an implicate role for FSH in spermatogenesis in the adult animal has yet to be identified (Sharpe, 1989).

1.2.3. Control of LH secretion and its actions.

Leutinising hormone (LH) acts exclusively on testicular Leydig cells and is the primary regulator of testosterone secretion. Testosterone has both paracrine actions and a host of peripheral effects in reproductive and non-reproductive organs (Sharpe, 1986), LH therefore represents the vehicle by which the brain is able to exert overall control of the timing of testosterone production. In addition, peripheral testosterone negatively regulates LH release from the pituitary.

LH stimulates testosterone synthesis and secretion through specific LH receptors located on the surface of Leydig cells. The regulation of testosterone synthesis is governed not only by peripheral LH levels but by the number of LH receptors (Sharpe, 1982). There is unequivocal evidence that LH (or hCG - an experimental substitute for LH) negatively regulates the number of its own receptors (Sharpe, 1984). Injections of LH or hCG into immature or adult intact or hypophysectomised rats causes a dose-dependent decrease in the number of available LH-receptors (Sharpe,1982). The physiological significance of the negative (or down regulation) of Leydig cell LH receptors remains uncertain. Down regulation however probably occurs under physiological conditions since repeated injections of small doses of LH, which mimic the normal pulsatile pattern of LH secretion, cause more pronounced receptor loss than when the same total dose of LH is given as a single injection (Sharpe & Mc Neilly, 1978). Leydig cells also possess spare receptors i.e. less than 1% of the available receptors need to be occupied to stimulate steroidogenesis maximally (Catt & Dufau, 1973; Mendelson *et al*, 1975), an arrangement which makes Leydig cells particularly sensitive to low hormone concentrations. The function of receptor down regulation might therefore be to reduce sensitivity to LH-stimulation without reducing the capacity of the Leydig cells to respond (Raff, 1976). Through this dynamic interplay of changing peripheral LH levels and LH receptor number testosterone production is maintained. In turn, testosterone acts at the level of the pituitary gonadotrophs to inhibit both LH and FSH secretion.

1.2.4. Testosterone.

Testosterone is synthesised from cholesterol through two separate pathways (Δ_4 and Δ_5). Within these pathways a cascade of reaction takes place with the formation of a number of intermediates requiring several enzymes

located within the smooth endoplasmic reticulum of Leydig cells (Eik-Nes, 1975; Chubb & Ewing, 1979; Ewing & Zirkin, 1983).

A number of recent studies have utilised the cytotoxic effects of ethane dimethane sulphonate (EDS) to investigate the role of the Leydig cell and its secretions, particularly testosterone, in spermatogenesis. EDS selectively perturbs Leydig cell function resulting in their complete destruction in the adult rat (Jackson *et al*, 1961, Kerr *et al*, 1985). Therefore the source of testosterone is removed. Without testosterone spermatogenesis breaks down progressively with the degeneration of germ cells (Bartlett *et al*, 1986). Spermatogenesis can be maintained by administering regular exogenous doses of long-acting testosterone esters which can maintain quatitative spermatogenesis for at least 10 weeks (Sharpe *et al*, 1988). During this 10 week period high systemic levels of testosterone are maintained resulting in almost complete suppresion of FSH and LH secretion. As a result Leydig cells do not regenerate. (For review of these experiments see Sharpe *et al*, 1990). Another finding was that in the absence of testosterone pachytene spermatocytes degenerate initially followed by round spermatids, both in stage VII. These effects are consistent with morphological data showing that deprivation of normal gonadotrophic stimulation either by hypophysectomy (Russell & Clermont, 1977), treatment with specific antisera to LH and FSH (Dym *et al*, 1977) or by a variety of steroidal agents known to severely reduce gonadotrophic secretion (Russell *et al*, 1981) results in the degeneration of pachytene spermatocytes and step 7 and step 19 spermatids present in stage VII. This adds further evidence in favour of the cyclical variations of testosterone in its effects on Sertoli cells and germ cells (Sharpe, 1983).

1.2.4.1. Paracrine effects of testosterone on Sertoli cells.

Testosterone acts on Sertoli cells and peritubular cells since only these cell types possess androgen receptors in addition to Leydig cells (Sharpe, 1982; 1983; 1986). Evidence in favour of the cyclical effects of testosterone on Sertoli cells are very convincing. The concentration of androgen receptors on Sertoli cells varies cyclically in accordance with the stage of the spermatogenic cycle (Parvinen, 1982). Coincident increases also occur in the rate of secretion of ABP at stages VII and VIII (Ritzen *et al*, 1982), and although the precise function of this protein is unclear (Ritzen *et al*, 1981) it is probably a transporter of testosterone capable of maintaining high testosterone levels within stage VII seminiferous tubules and in the microenvironment surrounding stage VII (Parvinen & Ruokonen, 1982). Further indirect evidence comes from the

ability of the seminiferous tubules to secrete a factor which inhibits the aromatase enzyme which converts testosterone to oestradiol. This enzyme is secreted in greatest quantities in stages VII-VIII (Boitani *et al*, 1981). For obvious reasons the physiological role of this factor is to minimise the aromatisation of testosterone at a stage when testosterone requirement is at its peak.

Despite clear evidence that testosterone is essential for spermatogenesis, its remains unknown how it exerts this effect. Its only known action is to stimulate ABP secretion (see Hansson *et al*, 1976). Recent experiments by Sharpe and collegues (1990 - unpublished data) suggest that, in the absense of testosterone in vivo, protein synthesis is decreased significantly, preferentially at stage VII- "the androgen dependent stage", but exactly how this relates to the maintenance of spermatogenesis remains to be established.

1.2.4.2. Paracrine effects of testosterone on peritubular cells and the vasculature.

Testosterone has other paracrine effects, most noticably on peritubular cells and the vasculature. Testosterone has been demonstrated to stimulate the production of P-Mod-S by peritubular cells, and this then acts on Sertoli cells to enhance the secretion of a number of Sertoi cell proteins including ABP and transferrin (Skinner & Fritz, 1986). However the physiological significance of these effects remains to be resolved.

Since the entry of blood-borne products for testicular utilisation and removal of waste is performed by the vasculature (and lymphatic vessels to a lesser extent) (Waites & Gladwell, 1982), the control of this system is essential. Blood borne products must pass through or between vascular endothelial cells and into testicular interstitial fluid before gaining access to the seminiferous tubules. It has now been demonstrate that the formation of interstitial fluid from serum is androgen regulated, presumably via effects of testosterone on the Sertoli cells and peritubular cells which then modulate the vasculature by secreting one or more vasoactive factors (Maddocks & Sharpe, 1989). More recently it has been shown that this effect of testosterone involves modulation of testicular blood flow (Damber *et al* - unpublished data). It is unknown whether these effects are localised to within the vicinity of stage VII tubules and similarly it is not known if these effects of testosterone are mediated only by stage VII tubules or by all stages of spermatogenesis (Sharpe, 1990).

1.3. REPRODUCTIVE TOXICOLOGY.
1.3.1. INTRODUCTION.

Over the past decade, many publications attest to the substantial increase in public and scientific concern regarding the potential of industrial and environmental chemicals to interfere with male reproductive function.

In the preceeding sections it will have become apparent to the reader that spermatogenesis is dependent on a series of complex biological interactions involving endocrine functions, the status of key testicular cells including the Sertoli and Leydig cells, and the paracrine interactions which exist between them. These mechanisms can be interrupted at various stages by chemical insult resulting in a decrease in fertility. A number of chemicals have been discovered to impair male reproductive function in routine toxicological screening tests. Furthermore, a number of these chemicals including 1,2-dibromo-3-chloro-propane (DBCP) and chlordecone have inadvertently been demonstrated as testicular toxicants in man (Whorton *et al*, 1977; Taylor *et al*, 1978) after being identified 10 to 15 years earlier in animal models as hazardous to fertility (Torkelson *et al*, 1961; Good *et al*, 1965; Huber, 1965).

The potential for a number of chemicals to disrupt or interrupt part of the integrated process of male reproduction is great. Compounds such as tetrahydrocannabinol affect the central nervous system, cadmium affects the vasculature, methyl chloride and Chloro sugars affect the epididymis (all indirect effects), whilst sulphasalazine affects sperm capacitation - an effect which occurs after the generation of sperm (Lamb & Foster, 1988). Gossypol, DBCP, the glycol ethers and phthalates (to name but a few) have direct effects on spermatogenesis. Other factors to be taken into account include the dose and duration of exposure and the pharmacokinetic properties of the compounds (their route of absorption, distribution characteristics, possible metabolism and elimination) (Dixon & Lee, 1980).

Interest in toxicants which have a direct effect on testicular function has grown since they present an opportunity to specifically disrupt spermatogenesis and study the roles of different cells in the process. An example of a compound used extensively as a biochemical tool to investigate the role of the Leydig cell and testosterone in spermatogenesis is ethane dimethane sulphonate (EDS) (Sharpe *et al*, 1989; 1990), the Leydig cell specific cytotoxin (Jackson *et al*, 1986). Although EDS was not discovered during toxicological testing it illustrates the potential of these compounds as tools for dissecting normal physiological processes. The potential for other testicular toxicants to have effects directly on other testicular cell types is also possible. For example

ethylene glycol monomethyl ether (EGME) is thought to have a direct effect on pachytene spermatocytes (Chapin *et al*, 1984). Due to the important role of the Sertoli cell in spermatogenesis the consequences of such an effect are often widespread and serious, at least in the short term. Sertoli cell perturbation results in gross germ cell degeneration, a reduction in testicular weight and temporary loss of fertility.

1.3.2. Techniques used to identify testicular disruption *in vivo*.

A number of *in vivo* and *in vitro* methods have been used to evaluate testicular function/dysfunction after chemical exposure. Those *in vivo* have included continuous breeding experiments (Lamb, 1988), the measurement of seminal parameters (primarily sperm function tests) (Zenick & Goeden, 1988), the use of serum hormones as an indication of pituitary and testicular function (de Kretser & Kerr, 1983), and morphological evaluation of the seminiferous epithelium (Chapin, 1988). Unfortunately, no useful or sensitive biochemical markers of testicular dysfunction have been identified to date. The potential for a marker would be enormous, reducing the work involved in reproductive toxicity studies and the number of animals used in these studies.

Of the *in vivo* techniques listed above, continuous breeding experiments represent a comprehensive test system for identifying a broad spectrum of male and female fertility problems within the context of regulatory authorities and industry. These are a 'front line' approach to testing the reproductive toxicity of compounds (Lamb, 1988). These tests have the additional advantage of identifying teratogenic effects and subsequent effects in the F_1 generation born to parents exposed to compounds. These tests are fundamental to reproductive toxicity testing but are outwith the scope of this thesis.

1.3.2.1. Evaluation of sperm function.

Evaluations of a number of parameters of sperm function can also be carried out on samples taken from ejaculates, vas deferens, or cauda epididymis. Recent techniques have been applied to evaluate serial ejaculates from rats pre-treated with compounds (Ratnasooriya *et al*, 1980; Zenick *et al*, 1984). End points of spermatogenic function for these samples include sperm count and concentration, assessment of sperm morphology and motility, the measurement of viability and the acrosome reaction. The sensitivity of these sperm parameters with respect to chemical disruption of spermatogenesis is questionable in some instances. Whilst sperm may well be damaged in transit through the epididymis

(e.g. by exposure to sulphasalazine) it is debatable whether a toxicant which affects spermatogenesis would have a direct effect on developing elongating spermatids aside from a 'wholesale' loss of different generations of germ cells. The detection of changes in sperm parameters in ejaculates is also preceded by a lag period in which disrupted germ cells must mature and be released and an additional 3 to 7 day epididymal transit time. Therefore, the loss of a specific generation of germ cells is detected as a decline in fertility some weeks later. For example, 2-methoxyacetic acid (MAA) selectively destroys pachytene spermatocytes, however, using parameters of sperm function and fertility as an index of spermatogenic disruption, the decrease in germ cell number was detected approximately 30 days after treatment (Ratnasooriya & Sharpe, 1989).

1.3.2.2. Evaluation of testis weight.

Because the mass of the testis is composed substantially of germ cells a characteristic feature is that, if there is severe toxicity for a period of time sufficient to induce significant germ cell loss, the weight of the testis falls proportionately. However in some circumstances significant tissue damage can also occur without a decline in testicular weight (e.g. Chapin *et al*, 1984). Therefore, in addition to testicular weight other indices of testicular function should be used, possibly the hormonal status of the animal (Chapin *et al*, 1982). Gross germ cell degeneration is normally accompanied by rises in peripheral FSH levels representative of Sertoli cell dysfunction (de Krester & Kerr, 1983), whilst changes in LH and/or testosterone (which rarely occur) would indicate Leydig cell dysfunction (Rehnberg *et al*, 1988). These fluctuations in peripheral hormonal levels are only detectable following gross disruption which can take a number of days to become manifest. These hormones, therefore, represent insensitive indices of testicular perturbation.

1.3.2.3. Evaluation of testicular morphology.

Morphological evaluation of the seminiferous epithelium is undoubtedly the primary technique for identifying testicular lesions (Chapin *et al*, 1983; 1984; 1985). Whilst histology cannot provide much information regarding the mechanism of action of a specific compound, it can identify the cells or region of tissue involved. The technique has a number of advantages and disadvantages. The primary consideration for histological preservation of such a complex organ as the testis is to maintain structural detail. Artifacts formed during histological processing and sectioning are often mistaken for chemically induced damage. Perfusion of the testes *in situ* is the best technique for fixation

to avoid artifacts (Kerr *et al*, 1984). This technique involves using the vasculature of the animal to circulate the fixative through the tissue. Testicular tissue may then be embedded in a suitable medium for sectioning. There are two suitable mediums for this purpose, epon araldite and glycomethacrylate (GMA). GMA is soluble to a number of water-based histological stains such as Periodic-acid Schiffs (PAS) and haematoxylin and eosin (H & E) and therefore allows a flexibility in the stains utilised (Chapin *et al*, 1985); however, GMA is expensive. Epon araldite is a relatively cheaper alternative. This medium allows sections cut thin enough to provide optimal resolution and minimal overlap of cells and structures, furthermore, the same block of tissue may be sectioned for light or electron microscopy without further processing. The major disadvantages with epon araldite are that, for light microscopy, epon araldite is soluble to only toluidine blue stain and also there is a limit to the size of the piece of tissue which may be fixed, embedded and sectioned (Chapin, 1988).

A number of considerations must be taken into account when utilising histology as a means of identifying the site and degree of damage produced by a compound. Often different compounds can have similar "end point effects " through initial effects on different cells (Chapin *et al.*, 1983; Creasy & Foster, 1984). Morphological evaluation of the seminiferous epithelium at a point when germ cell degeneration is at its peak does not identify the initial cell(s) damaged, only the degree of effects. Serial kill schedules should therefore be adopted particularly at time points immediately after dosing to pinpoint morphologically the initiation of a lesion. For example, Creasy *et al* (1983) using electron microscopy identified Sertoli cell vacuolation 3 hours after phthalate administration. The ramifications of the initial cellular perturbation on subsequent morphology (and function) can then be followed serially at later time points.

Histology imposes a number of constraints on any experimental protocol. To obtain high resolution from quality fixation, the animal must be killed. It is therefore impossible to follow the generation and progression of a testicular lesion taking serial tissue samples from the same animal. Thus the generation and progression of a lesion must be followed in groups of animals killed at serial time points after dosing.

1.3.3. Techniques used to identify testicular disruption *in vitro*.

Following the histological identification of the cell(s) perturbed it is then possible to isolate and investigate the mechanisms of toxicity *in vitro*. In recent years rapid growth has been made in the application of *in vitro*

methodology for the evaluation of chemical toxicity. Progress has been made in certain areas, whilst others are still in the early stages of developments. Due to the complexity of spermatogenesis, developing a culture system structurally representative of that *in vivo* is extremely difficult, particularly using primary cell cultures.

Single cell-type cultures of Sertoli cells and Leydig cells have been generated and used to evaluate the toxicity of chemicals (Gray, 1986; 1988). A more physiological rationale is to co-culture testicular cell types such as Sertoli cell - germ cell , Sertoli cell - Leydig cell and possibly Sertoli cell - germ cell - Leydig cell combinations. Modifications to these cell culture systems have included the addition of extracellular matrix (Skinner & Fritz, 1986) to the Sertoli cell culture and the use of bicameral chambers (Byers *et al*, 1986). However, as these systems increase in complexity so the problems associated with the study of toxicant action also increase proportionately.

1.3.3.1. Evaluation of cell function *in vitro*.

Following the initial identification of Sertoli cell and/or germ cell toxicant induced perturbation *in vivo* using histology, Sertoli cell - germ cell co-culture systems have been adopted by a number of testicular toxicologists to study chemically induced testicular disruption *in vitro*. Compounds investigated in this manner have included the phthalates (Gray *et al*, 1984), glycol ethers (Gray *et al*, 1985) and *meta*-dinitrobenzene (Foster *et al*, 1987).

A number of indices of function have been used to evaluate the effects of toxicants on isolated testicular cells and co-cultures. These have included histology, germ cell exfoliation in co-culture and biochemical measurements including lactate and pyruvate production for Sertoli cell monocultures and Sertoli cell-germ cell co-cultures and the stimulation or inhibition of steroidogenesis in Leydig cell cultures. Again histology at the light and electron microscopic level has a key role to play in the further indentification of toxicant induced cellular damage *in vitro*. A number of effects have been identified histologically in Sertoli cell-germ cell co-cultures following toxicant perturbation. These include Sertoli cell vacuolation, germ cell vacuolation (Williams & Foster, 1988), phagocytosis of germ cells by Sertoli cells (Foster *et al*, 1987) and a number of ultrastructural changes (Creasy *et al*, 1983). Comparison of histology at the light and electron microscopic level have also been made on cultures in order to determine the target cell perturbed by the toxicant. The technique at the ultrastructural level is particularly good in

identifying not only the target cell but also the duration between exposure and the formation of an intracellular lesion.

Gray and Beamand (1984) examined the effects of a range of toxicants on Sertoli cell-germ cell co-cultures using cell detachment as an index of perturbation. A number of compounds produced germ cell detachment in the system, this was dependent on the cell type affected and the dose administered to the cultures. The technique represents a simple index of Sertoli cell and germ cell function (Gray, 1986). However, care must be taken in selecting the correct dose levels equivalent to those observed *in vivo* since some compounds (e.g. Hydroxy urea and cyclohexylamine) produce cell exfoliation at overtly cytotoxic concentrations (Gray & Beamand, 1984). In addition, the metabolism of the parent compound *in vivo* must be taken into consideration. The parent compound may not be the toxicant, however a metabolite(s) produced in the testes or other organ(s) may be more toxic e.g. EGME and its metabolite MAA (see Chapter 6). Therefore the technique of measuring cellular exfoliation has been adopted by a number of investigators as a crude index of Sertoli cell function (Foster *et al,* 1987 - to examine the effects of *meta* -dinitrobenzene *in vitro*), and an index of germ cell function i.e. to examine the effects of glycol ethers, trifluoroethanol and a number of other germ cell toxicants.

A number of Sertoli cell biochemical functions and secretions have also been used as end points of toxicity *in vitro*, these include lactate and pyruvate. Williams & Foster (1988) used lactate and pyruvate production by Sertoli cells as a measure of cellular perturbation following the administration of the Sertoli cell toxicant *meta* -dinitrobenzene (mDMB). The concentration of both lactate and pyruvate in the medium increased dose-dependently following mDNB administration. Beattie *et al* (1984) has also reported that MAA inhibited lactate production by cultured rat Sertoli cells and suggested that this may be the mechanism by which MAA produces spermatocyte injury *in vivo*. Furthermore, the addition of lactate supplementation to the cultures did not protect against the toxicity of the compound (Beattie *et al,* 1985). In contrast, phthalate esters (which are also Sertoli cell toxicants) caused a stimulation of Sertoli cell lactate production but only when the active metabolites of the parent compound were used over a concentration range similar to that measured *in vivo* (Moss *et al,* 1985; 1986). Pyruvate levels also vary following toxicant exposure thus altering the ratio of pyruvate to lactate. These low molecular weight substances therefore represent biochemical indices of Sertoli cell function *in vitro*, however, whether these changes occur *in vivo* and are the cause of the subsequent germ cell degeneration (as has been suggested for MAA - Beattie *et*

al, 1984; 1985) is impossible to ascertain with current techniques. In addition to lactate and pyruvate, Sertoli cells also secrete a wide range of proteins into the culture medium (Griswold, 1988). The ability of Sertoli cells to survive in serum free medium makes the analysis of secretory proteins and their application as possible end points of Sertoli cell toxicity a feasible proposition. ABP is probably the best characterised of these proteins and its use as a functional marker enables observations made *in vitro* to be correlated with corresponding measurements made *in vivo* (Spitz *et al*, 1985). Noticeable changes in ABP production are also measured following the attachment or de-attachment of germ cells from Sertoli cell monolayer cultures (Galdieri *et al*, 1984; Jegou *et al*, 1988). Other Sertoli cell products used as indices of dysfunction include transferrin whilst the use of two dimensional polyacrylamide gel electrophoresis to evaluate changes in the patterns of protein synthesis following perturbation has been suggested (Lamb & Foster, 1988).

A more physiological approach to studying testicular toxicity *in vitro* is to use isolated seminiferous tubules. These cultures are potentially useful since they combine maximal retention of *in vivo* architecture and function (compared to isolated cell preparations) with the advantages of *in vitro* systems in terms of controlled experimental conditions. Again a number of end points may be used as indices of toxicity including histology and the measurement of Sertoli cell secretory proteins. To date the system has been used sucessfully by reproductive physiologists (Parvinen, 1982) but not by toxicologists. The possible reasons for this included the relative complexity of the culture system when studying cell specific toxicity and the time and effort required to mechanically dissect the tubules. The potential usefulness of this culture system for toxicological evaluation of compounds *in vitro* therefore remains to be assessed.

1.4. Aims of the studies in this thesis.

The aims of the studies presented in this thesis were three-fold: Firstly, to use known chemical testicular toxicants as biochemical tools to disrupt normal testicular function in adult rats and testicular cultures derived from rats. Specifically, to disrupt spermatogenesis through Sertoli cell (i.e. directly) and/or germ cell (i.e. indirectly) dysfunction and to observe the development and regression of time- and stage- dependent lesions and their relationship to morphological and functional changes both *in vivo* and *in vitro*. Secondly, to generate and validate a new and potentially useful *in vitro* male reproductive toxicity screening system by comparison to the established cell

culture system and *in vivo* rat model. In particular the use of seminiferous tubules isolated from toxicant pre-treated rats at serial time points after dosing. Thirdly, to assess the usefulness of inhibin as a biochemical marker of testicular perturbation both *in vivo* and *in vitro* with a view to its use in male reproductive toxicity testing to identify the target and degree of disruption to spermatogenesis. With the recent development of inhibin radioimmunoassays it is now possible to measure this protein in body fluids. Historically, FSH has been postulated to control inhibin secretion in a classical negative feedback manner. Severe testicular dysfunction has always been associated with raised blood levels of FSH and this has always been inferred as evidence for decreased secretion of inhibin (de Kretser & Kerr, 1983). Therefore, a relationship between inhibin, FSH and testicular dysfunction has already been established. However, FSH secretion represents a rather insensitive index of testicular dysfunction since levels only increase after very severe disruption of spermatogenesis (de Kretser & Kerr, 1983). Since the radioimmunoassay has become available the measurement of inhibin directly as another, potentially more sensitive indicator of Sertoli cell dysfunction in comparison to FSH has been investigated *in vivo* and *in vitro*.

CHAPTER 2.

MATERIALS AND METHODS.

2.1. Animals and treatments.

The rats used in these studies were either of the Sprague-Dawley derived strain from Charles River Ltd (U.K.) bred in the Unit of Reproductive Biology in Edinburgh, or of the Alpk:AP (Wistar derived) strain from the I.C.I. Animal Breeding Unit, Alderley Park, Macclesfield, Cheshire, U.K. They were housed under conventional conditions (light:dark cycle 12h:12h) at a temperature of 21°C, food and water were available *ad libitum*.

All compounds or vehicles were administered by oral gavage (see relevant sections for experimental protocol which includes details of rat strains, compounds and doses used).

2.2. Collection of body fluids for hormone measurement.

2.2.1. Interstitial Fluid (IF).

The collection of IF from rat testes were performed using techniques described by Sharpe & Cooper (1983). Basically rats were killed by inhalation of CO_2 followed by cervical dislocation, the testes were isolated immediately after death, blotted to remove excess blood and a small incision made at the caudal end of the testicular capsule. The testis was then placed in a preweighed 83 x 13mm polystyrene tube such that it was suspended 1-2 cms above the tube bottom. IF was allowed to drain from the testis for approximately 16h at 4°C. Testicular weight was recorded and the testicular IF was centrifuged (1000g) for 15 min at 4°C to precipitate contaminating erythrocytes. IF volume was then determined by aspiration in measured volumes and an aliquot taken and diluted 1:10 with M199 containing Hanks salts and 20mM Hepes and stored at -20°C. Contamination of collected IF with testicular blood was variable, but from the known testicular blood content (Setchell & Sharpe, 1981) and the amount removed by blotting during testicular isolation, this contamination was estimated to be always <10%.

2.2.2. Serum.

Blood was obtained at death from the severed jugular veins and placed in non-heparinised collection tubes. Following storage at 4°C for 16 to 24h the serum was separated by centrifugation (1500g) for 30 min at 4°C. The serum was aspirated from the tube and stored at -20°C.

2.3. MEASUREMENT OF CIRCULATING HORMONE LEVELS.

2.3.1. FSH and LH Radioimmunoassay.

Serum levels of FSH and LH were measured using a method previously described by Fraser & Sandow (1977) incorporating a double-antibody radioimmunoassay technique based on kits supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), U.S.A.

For FSH: Standard curves were set up in each assay using a rat FSH stock solution (RP-2) provided in the kit. The concentration of standards ranged from 250 ng/ml double diluted to 3.9 ng/ml. Standards and serum samples were diluted with assay buffer (0.01M phosphate buffered saline, pH 7.4 (PBS) containing 1% bovine serum albumin (crude grade- Sigma, Poole, Dorset, U.K.) to reduce non-specific binding and 0.01% thiomersalate (B.D.H.) as an antibacterial agent. To the diluted standard or serum samples (total volume 400 µl), 100 µl of the first antibody (rabbit anti rat FSH-S-11 - provided in the NIAMDD kit) was added at a final dilution of 1:2000; this dilution was made in a buffer containing 0.01M phosphate buffered saline, 0.05M ethylenediaminetetra acetic acid (EDTA) and 0.5% normal rabbit serum (Scottish antibody production unit, Law Hosptial, Lanarkshire, Scotland). This mixture was vortexed and stored for 24h at 4°C. Next, ^{125}I labelled rat FSH (NIAMDD) was diluted in assay buffer to produce approximately 15,000 cpm/100 µl of solution and 100 µl added to each tube, vortexed and stored at 4°C. (The rat FSH was radioiodinated by Miss Irene Cooper using the Chloramine-T method). After a further 24h, the precipitating antibody was added. This was a donkey anti rabbit serum (DARS - Scottish antibody production unit) used at a final dilution of 1:15 and diluted in assay buffer. Following a further 24h at 4°C 1ml saline was added to each tube and centrifuged (2500g) for 30 min at 4°C. The supernatant was poured off and the precipitate containing the radiolabel was counted for gamma emission using a gamma counter (NE 1600 - Nuclear Enterprises). FSH assay sensitivity (90% B/B_0 = [amount bound - non-specific binding] / [total bound - non-specific binding]) was in the range of 3 to 6 ng/ml and intra- and inter- assay coefficients of variation were 5 and 9 ng/ml respectively. Serum samples assayed for FSH from both treated and control rats for every complete experiment were assayed simultaneously.

For LH: Standard curves were set up in each assay using a rat LH stock solution (RP-2) provided in the NIAMDD kit. The concentration of standards ranged from 100 ng/ml double diluted to 0.78 ng/ml. Standards and

serum samples were diluted with assay buffer (0.01M phosphate buffered saline, pH 7.4 containing 1% bovine serum albumin (crude grade) and 0.01% thiomersalate). To the diluted standards or serum samples (total volume 400 µl), 100µl of the first antibody (rabbit anti rat LH-S-7 - also provided in the NIAMDD kit) was added at a final dilution of 1:4000; this dilution was made in buffer containing 0.01M phosphate buffered saline containing 0.05M EDTA and 0.5% normal rabbit serum (Scottish antibody production unit). The mixture was vortexed and stored for 48h at 4°C. Next, ^{125}I labelled rat LH (NIAMDD) was diluted in assay buffer to produce approximately 10,000 cpm/100 µl of solution and 100 µl added to each tube, vortexed and stored for 24h at 4°C. (The rat LH was radioiodinated by Miss Irene Cooper using the Chloramine-T method). The method continued as for FSH above. LH assay sensitivity (90% B/B_o) was in the range of 9 to 14 ng/ml and intra- and inter- assay coefficients of variation were 6 and 9 ng/ml respectively. Serum samples assayed for LH from both treated and control rats for every complete experiment were assayed simultaneously.

For both assays results were calculated using an in house log-logit transformation computer program ('Assay Zap' - Dr.P.Taylor) run on an Apple-Macintosh computer.

2.3.2. Testosterone measurement.

Serum and IF levels of testosterone were measured using a ^{3}H-based radioimmunoassay previously validated by Corker & Davidson (1978). Serum samples were extracted with hexane:diethyl ether whilst IF samples were assayed directly after appropriate dilution (Sharpe & Cooper, 1983).

For serum samples, tritiated testosterone, approximately 1000 cpm in a volume of 20 µl, was added to 100 µl of plasma in duplicate. This was extracted with 1.5mls redistilled hexane-diethyl ether (4:1). After vortex mixing for 5 mins the aqueous phase was frozen in a solid CO_2- ethanol bath. The organic phase was decanted, dried down under nitrogen gas and reconstituted in 200 µl of assay buffer. Of this, 50 µl was used to determine percentage recovery of individual samples whilst 100 µl was used in the assay. Quality control plasma samples were extracted and assayed in an identical manner.

Testosterone standards were prepared from a solution containing 1 µg testosterone in 1ml ethanol. Of this solution 640 µl were evaporated to dryness over nitrogen vapour and the residue reconstituted in 100 µl assay buffer (see below for composition) to give a concentration of 640 pg/100 µl.

Standard curves were set up using this stock solution. Standards ranged from 320 pg/100 µl double diluted to 5 pg/100 µl. Standards and testicular IF samples were diltued with assay buffer (0.01M phosphate buffered saline, pH 7.0 containing 0.1% gelatin and 0.01% thiomersalate). To 100 µl of standard or sample, 100 µl of the first antibody (EO-1 - raised against testosterone-3-carboxymethyloxime-BSA in the goat [Free & Tillson, 1973]) was added at a final dilution of 1:6000 in assay buffer, (mixed together in a glass tube) in addition to 100 µl of tracer, ^{3}H testosterone (Amersham International, Kent, U.K.), containing approximately 8000 cpm/100 µl. This mixture was either vortexed and stored at room temperature for 1h or stored at 4°C for at least 24h. Separation of bound and free testosterone was made by incubating each tube with 1ml of dextran-coated charcoal for exactly 15mins in ice, (0.25% Norit-A charcoal -Sigma and 0.025% dextran T 70 -Pharmacia, U.K. made up in assay buffer). After 15 mins all tubes were centrifuged (2500g) for 10mins at 4°C and the supernatant was decanted into scintillation vials, mixed with 3.5mls scintillation fluid (RIA Luma, Lumac, The Netherlands) and counted for beta emissions using a beta counter (LKB, Wallac, 1209). Testosterone assay sensitivity (90% B/B$_o$) was in the range of 8 to 20 pg/ml and intra- and inter-assay coefficients of variation were 12 and 28 pg/ml respectively. Serum and IF samples assayed for testosterone from both treated and control rats for every complete experiment were assayed simultaneously. As stated earlier, results were calculated by log-logit transformation using the Assay Zap program.

Recent data suggests that the IF testosterone levels are overestimated due to the continued production of testosterone by Leydig cells *in situ* during testicular IF drip collection (Maddocks & Setchell, 1988; Sharpe *et al*, 1988) (see Table 2.1. page 51). However, whilst the absolute levels of testosterone in IF are therefore overestimated, the pattern of change of testosterone levels in IF and peripheral serum have been demonstrated to remain identical in a number of experiments (Sharpe *et al*, 1988). In view of the fact that testosterone levels were measured in both IF and serum samples taken from each animal during the course of each experiment the quantitative levels may not be exactly correct but the pattern of change between each animal, in each group and throughout each experiment remained constant. Therefore these quantitative differences were ignored.

2.3.3. Inhibin Radioimmunoassay.

Inhibin was measured using a double-antibody radioimmunoassay based on an antibody (S55) generated in a sheep to the 1-26

sequence of the N-terminus of the α subunit of porcine 32 KDa inhibin (Sharpe et al., 1988). This material ([1-26] α plus Glycine [27] Tyrosine [28]) was synthesised and provided kindly by Dr.J. Rivier of the Salk Institute, U.S.A. Standard curves were set up in each assay using a [1-26] α-inhibin stock solution from the same source. The concentration of standards ranged from 1000 pg/ml double diluted to 1.95 pg/ml using assay buffer. Samples and standards were diluted in 0.1M phosphate buffered saline, pH 7.4 containing 1% BSA (crude grade) and 0.01% thiomersalate. To the diluted standards or sample (total volume 400 μl), the first antibody (S55) was added at a final dilution of 1:20,000 in assay buffer, vortexed and stored at 4°C for 24h. Next, ^{125}I-labelled porcine inhibin was diluted in assay buffer to produce approximately 15,000 cpm/100 μl of solution and 100 μl added to each tube, vortexed and stored at 4°C. (The [1-26] α-inhibin was radioiodinated by Dr Bruce Campbell or Miss Wendy Crow using the Chloramine-T method). After a further 24h, 100 μl of normal sheep serum (Scottish antibody production unit) was added to each tube at a dilution of 1:600 in assay buffer together with the precipitating antibody - donkey anti sheep goat (Scottish antibody production unit) at a final dilution of 1:10 in assay buffer. Following a further 24h at 4°C 1ml of saline was added to each tube and centrifuged (2500g) for 30 min at 4°C. The supernatant was poured off and the precipitate containing the radiolabel was counted for gamma emission using a gamma counter (NE 1600 - Nuclear Enterprises). Inhibin assay sensitivity (90% B/B_0) was in the range 3-8 pg [1-26] α-inhibin or 6-16 mU equivalent of an ovine rete testis fluid (oRTF) standard with an arbitary potency of 1 U/mg (Eddie et al., 1979) provided kindly by Dr.J.K. Findlay, Melbourne, Australia.

Testicular IF, spent media from rat seminiferous tubules cultured for 48h in the presence of 50ng rat FSH-I-3/ml (STCM) and Sertoli cell-conditioned culture medium (SCCM) were run as double dilutions in the assay. These samples gave displacement curves in the radioimmunoassay which paralleled that of unlabelled [1-26] α-inhibin and the oRTF standard (see Fig 2.1). Results were calculated using log-logit transformations. The intra- and inter-assay coefficients of variation were <10%.

In addition to the radioimmunoassay, samples of testicular IF and STCM were assayed in an in vitro inhibin bioassay, using sheep pituitary cells (Tsonis et al, 1986). Following the removal of endogenous steroids by incubation for 1h at 21°C with 1 mg/ml dextran-coated charcoal, pools of testicular IF or STCM were added at multiple doses to the pituitary cells. These samples gave a dose related inhibition of FSH release and their inhibin content
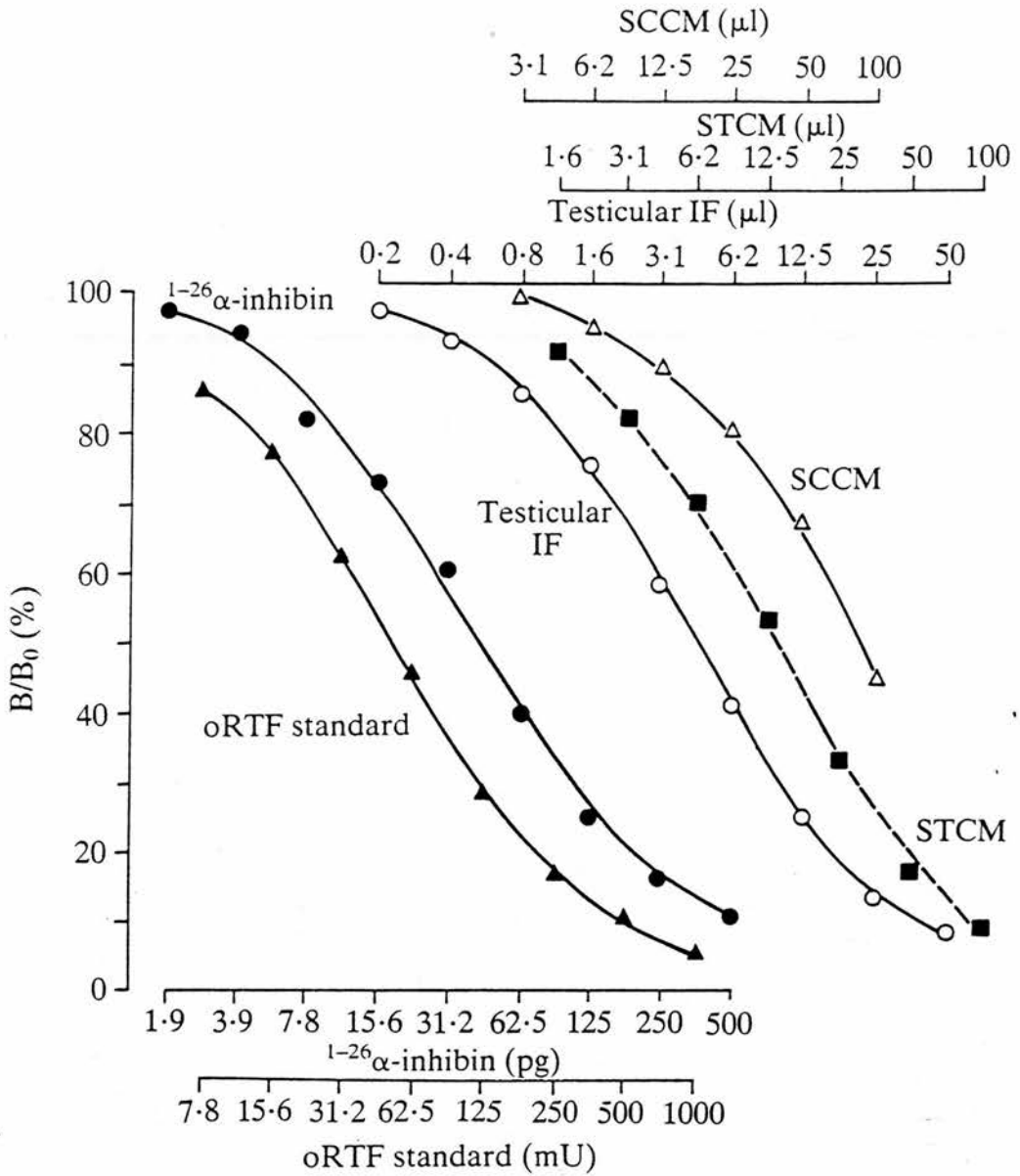
Fig 2.1. Radioimunoassay for $^{1-26}\alpha$-inhibin: displacement curves are shown for unlabelled $^{1-26}\alpha$-inhibin, an ovine rete testis fluid standard (oRTF) or inhibin standard and dilutions of rat testicular interstitial fluid (IF), rat Sertoli cell-conditioned culture medium (SCCM) and rat seminiferous tubule-conditioned culture medium (STCM).

was computed by standard bioassay methods with reference to an oRTF standard (1 U/mg; see Tsonis *et al*, 1986 for details). Bioassays were kindly performed by Miss Rose Leask.

Testicular IF was collected for inhibin measurement using the drip collection technique. Because testosterone is continually produced during drip collection inhibin may be produced in a similar manner. Therefore the levels of IF inhibin collected from the same testis were measured for comparison at 10 min versus 16h. No consistent difference was detected in immunoreactive inhibin between the two time points in samples collected from rats of different ages (see table below).

Table 2.1. Comparison of the levels of testosterone and $^{1-26}\alpha$-inhibin in testicular interstitial fluid (IF) collected at 4°C over 10 min or 16h from the same testis of rats of various ages.

Age of rats (days)	No. of rats	Parameter (units)	Level in testicular IF collected from same testis over:	
			10min	16h
160	8	Testosterone (nmol/l)	624 ± 372	1234 ± 555 **
135	6	$^{1-26}\alpha$-inhibin (ng/ml)	10.2 ± 2.0	12.0 ± 1.6 *
160	4	$^{1-26}\alpha$-inhibin (ng/ml)	10.1 ± 2.5	9.5 ± 0.9 NS
35	8	$^{1-26}\alpha$-inhibin (ng/ml)	75.8 ± 11.3	74.3 ± 12.3 NS

Value are means ± SD; **$P<0.001$,*$P<0.02$, compared with values for 10 min (paired t test). NS = not significant.

Additional evidence that inhibin does not leak from the seminiferous tubules during overnight IF collection is apparent from the fact that IF levels are only a small fraction (approximately 1%) of intratubular levels.

Because levels measured in seminiferous tubule-conditioned medium following repeated freeze thawing showed some variation, all samples (IF and culture medium) were analysed for inhibin content after just one episode of freeze-thawing.

For reasons which are currently unclear, it is not possible to accurately measure inhibin levels in rat serum probably due to interference or cross-reaction of factors resulting from the clotting process. Thus serum values are always 3- to 10- fold higher than levels measured in plasma and show no meaningful relationship to other biological parameters such as FSH (R.M. Sharpe & S. Maddocks, unpublished data). In contrast, levels of inhibin

measured in plasma by the [1-26] α-inhibin RIA show excellent agreement with results obtained from the inhibin bio-assay (Maddocks & Sharpe, 1989) and in most instances make 'biological sense' eg plasma levels change dramatically with age in parallel with changes in the IF levels of inhibin and the plasma levels of FSH (Maddocks & Sharpe, 1990).

2.4. HISTOLOGY.

2.4.1. Perfusion-fixation.

The perfusions were performed using techniques described previously by Kerr *et al*, (1984). Rats were anaesthetized using ether, the abdominal cavity was opened and the descending aorta located and cannulated approximately 1.0 cm below the heart. The ascending vena cava or left ventricle of the heart was then severed and 0.9% saline containing 5 U/ml heparin perfused by gravity feed (3 foot height) via the cannula at a rate of 5 to 10 mls/min until the testicular blood vessels had cleared of blood. Animals were then perfused with fixative solution (0.17M cacodylate buffer, 2% paraformaldehyde, 0.26M picric acid plus 3% glutaraldehyde, pH 7.4, approximately 500 mOsm) at a rate of 2-4 mls/min for 30 to 45 min until the testes were hard. The testes and epididymides were then dissected from the animal.

The testes were decapsulated and the equatorial region cut into 2 mm thick slices using a razor blade. These were then trimmed to a square shape and then diced into cube-shaped blocks of approximate dimensions 2 x 2 x 2 - mm. These blocks and the epididymides were placed into the same fixative solution as used for perfusion for 2 to 48h at 4°C before primary processing as described below.

2.4.2. Processing of perfusion fixed testicular tissue.

Blocks of testicular tissue were processed through the following schedule:

1. Washed several times in 0.2M cacodylate buffer for a minumum of 4h.
2. Post-fixed in 2% osmium tetroxide (in 0.2M cacodylate) for 2 to 3h at 4°C.
3. Washed several times in 0.2M cacodylate buffer for a minimum of 30 min.
4. Washed twice in 0.05M maleate buffer each for a minimum of 15 min.
5. Post-fixed in 1% uranyl acetate at room temperature for 2h.
6. Washed twice in 0.05M maleate buffer each for a minimum of 15 min.

7. Washed twice in 70, 80, 90 and 95% ethanol, each wash lasting 10min.

8. Washed several times in 100% ethanol for 60 min.

9. Washed twice in neat propylene oxide, each for 30 min.

10. Pre-embedded in a 50:50 mixture (v/v) propylene oxide/epon araldite overnight (24h).

The 50:50 mixture was then poured off and the blocks placed into an oven at 60°C for 10 min to evaporate excess propylene oxide. The blocks were then placed into fresh liquid araldite which was changed twice, each for a minimum of 6 and 12h. Following this the blocks were embedded in fresh liquid araldite in plastic cylindrical flat bottomed capsules. These were placed in an oven at 60°C for 24 to 36h to allow the araldite to polymerize.

2.4.3. Processing of perfusion-fixed epididymal tissue.

The epididymides were washed serveral times in 0.2M cacodylate buffer for a minimum of 4h. Each epididymis was then sectioned in the caput, caudal and mid-regions to yield transverse slices. These were placed into holders and processed through the following schedule:

1. Washed in 70% alcohol for 1.5h.

2. Washed in 80 and 90% ethanol each for 1h.

3. Washed twice in 99% industrial methylated spirits each for 1h.

4. Washed twice in 100% ethanol for 1.5h.

5. Washed three times in xylene each for 1.5h.

6. Dipped twice in wax for 1.5h.

7. Dipped in wax then placed under vacuum for 20 mins.

8. Dipped in wax and allowed to set.

2.4.4. Sectioning.

Following the removal of excess araldite from around the blocks of testicular tissue using a hacksaw, semi-thin sections were cut to a thickness of 0.5 to 0.75μm using glass or diamond knives on a Reichert Jung microtome (Model No. 2050). Epididymal sections were cut to a thickness of 3 to 4μm using a hand-operated microtome and D-profile knife.

2.4.5. Staining.

Sections of testicular tissue were stained using toluidine blue at approximately 60°C for a few seconds; the toluidine blue was always freshly

filtered before use. Sections of epididymal tissue were stained using haematoxylin and eosin.

2.4.6. Microscopy and photography.

Sections were observed and photographed using a Zeiss photomicroscope.

2.5. CELL CULTURE.

2.5.1. Preparation of primary testicular cell cultures.

The method used was a modification of the technique described by Gray & Beamand (1984). Male rats (28 to 30 days old - 70 to 110g bodyweight - Alpk:Ap strain from I.C.I.) were killed by an overdose of halothane followed by cervical dislocation. The testes were dissected and decapsulated in Hanks Basic Salt Solution (BSS) (without calcium, magnesium or phenol red). The testicular tissue was then cut into pieces of approximately 5mm cubed and incubated in 0.25% w/v trypsin solution containing 10μg/ml DNAase at 32°C for approximately 15 min, shaking vigorously every few mins to facilitate the separation of tubules. The solution was then passed through a 75μm filter and the tissue on the filter washed off in Hanks BSS. These seminiferous tubule pieces were resuspended in Hanks BSS containing 0.1% w/v collagenase and incubated at 32°C for approximately 8 min, shaking vigorously every few mins to facilitate the breakdown of tubules into small aggregates of Sertoli cells and germ cells. The solution was then passed through a 45μm filter and the tubule fragments retained on the filter were washed again in Hanks BSS. This tissue was placed into a 50ml measuring cylinder, the filter was then inverted and back-washed with culture medium (Eagles minimum essential medium (MEM) plus 0.1 mM non-essential amino acids, 4.0 mM L-glutamine, 50U penicillin and 50μg streptomycin/ml). This back-washed culture medium was poured into the measuring cylinder. Foetal bovine serum (10%v/v) was then added to the cylinder which was agitated vigorously for 30 secs. A sample of this solution was diluted 1:10 with sterile saline and pipetted a number of times to separate aggregates into single cells. Following this, 100μl of 0.05% trypan blue was added, mixed, and the cells counted using a haemocytometer. Blue staining cells were considered to be non-viable. The cells in the main preparation were diluted using culture medium containing 10% foetal bovine serum to a density of 1×10^7 viable cells/1.5mls/well in a 6-well culture plate. These were incubated at 32°C in a humified atomosphere of 95%

air/5% CO_2. The culture medium was replaced every 24h (foetal bovine serum was present during the first 24h only). The resulting cell cultures consisted of a mixture of Sertoli cells and germ cells (mainly pachytene spermatocytes and round spermatids) which are referred to hereafter as Sertoli cell-germ cell co-cultures. These cultures contained peritubular cells, however since a more physiological environment was required *in vitro* to approximate that *in vivo* no effort was made to reduce the numbers of contaminating peritubular cells which only comprised approximately 5% or less of the total cells on the plate. After 72h the co-cultures were treated with toxicants as described in Chapters 3, 4 and 5.

Sertoli cell only cultures were generated from the co-cultures described above using the method of Galdieri *et al* (1981). Sertoli cell-germ cell co-cultures were treated with hypotonic tris (hydroxymethyl) aminomethane hydrochloride (20mM, pH 7.4) for 5 mins 48h after the initial plating to remove germ cells. The Tris was then aspirated and replaced with culture medium and, following a further 24h period, cultures were treated with toxicants. This Tris treatment method results in the removal of >90% of the germ cells and had only transient effects on Sertoli cell morphology and function.

2.5.2. Histological examination of cultures.

Glass coverslips were placed into 6-well plates during the initial cell-plating procedure. Following a 24h exposure period of cultures to a specified toxicant the coverslips were fixed with Bouin's fluid and stained using haematoxylin and Papanicolou's stain for histological examination. The fixing and staining procedures were as follows:

1. Fixed in Bouin's solution for 20 mins.
2. Rinsed in tap water until clear.
3. Stained using Harris's haematoxylin for 5 secs.
4. Rinsed in tap water until blue.
5. Washed in 70% ethanol for 30 mins.
6. Washed in 95% ethanol for 30 mins.
7. Counter-stained using Papanicolou's stain (EA65) for 2 mins.
8. Rinsed in 95% ethanol for 30 mins.
9. Rinsed in absolute ethanol for 30 to 60 mins.
10. Rinsed in xylene for 30 mins.
11. Removed from xylene, blotted and mounted on a glass slide using DPX mounting fluid.

2.5.3. Measurement of cellular exfoliation from Sertoli-germ cell co-cultures.

The measurement of cellular exfoliation (mainly germ cells) from testicular Sertoli cell-germ cell co-cultures has proved to be a useful, if crude, index of toxicant action in co-culture (Gray & Beamand, 1984; Foster *et al*, 1987). Cellular exfoliation was monitored 24h after exposure of co-cultures to toxicant using a Coulter counter and techniques previously validated by Gray & Beamand (1984) and Foster *et al*, (1987). Basically, the culture medium from each well was diluted 1:10 with Isoton(TM) solution. The Coulter counter withdraws 500µl from the sample through a photoelectric cell (window) and counts particulate material (mainly cells) passing through this window. Duplicate determinations were made for each sample.

Exfoliated cells were considered to be primarily germ cells since treatment of co-cultures with a toxicant at a toxic concentration resulted in significant exfoliation of cells whereas in comparison an insignificant number of exfoliated cells were measured in Sertoli cell only culture medium following the addition of an identical dose of the same toxicant (see relevant sections for details). In the latter instance the insignificant number of exfoliated cells is presumed to represent the detachment of residual germ cells that contaminate Sertoli cell cultures following Tris treatment.

The viability of cells exfoliated after toxicant exposure was estimated by incubating aliquots of these cells in 0.05% trypan blue dye and counting the number of cells which absorbed the dye. Doses of toxicants which resulted in >30% of exfoliated cells absorbing the dye were considered to exhibit non-specific cytotoxicity.

2.5.4. Measurement of lactate and pyruvate secretion by Sertoli cells in culture.

The measurement of lactate and pyruvate secretion by Sertoli cells in culture has been used as an index of Sertoli cell secretory function by previous investigators (Williams & Foster, 1988). These investigators have used the amounts of both moeities secreted in culture as an index of function following perturbation by toxicants. In this thesis lactate and pyruvate secretion by Sertoli cells in culture following toxicant treatment has also been used as an index of secretory function.

The concentration of lactate and pyruvate were measured in culture medium using the methods of Gutman & Wahlefeld (1974) and Czok and Lamprecht (1974) respectively and modified by Williams & Foster (1988).

These assays are based on the use of lactate dehydrogenase (LDH) and NAD or NADH, the endpoint involving monitoring the change in absorbance at 340nm.

Principle of the assays:

Lactate:-

$$\text{Lactate} + \text{NAD} + \text{hydrazine} \xrightarrow[\text{1 hr at 25}^\circ\text{C}]{\text{LDH at pH 9.0}} \text{Pyruvate/hydrazine} + \text{NADH}$$
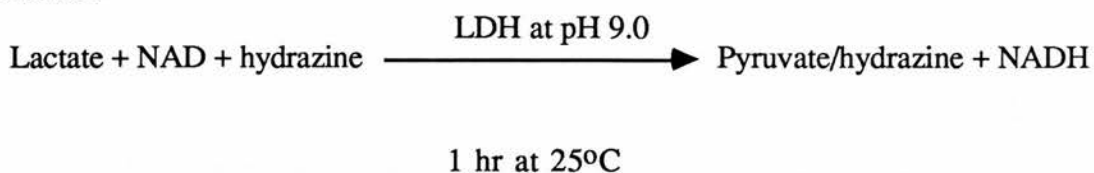
Follow the **increase** in absorbance at 340nm.

Pyruvate:-

$$\text{Pyruvate} + \text{NADH} \xrightarrow[\text{3 mins at 25}^\circ\text{C}]{\text{LDH at pH 7.6}} \text{Lactate} + \text{NADH}$$
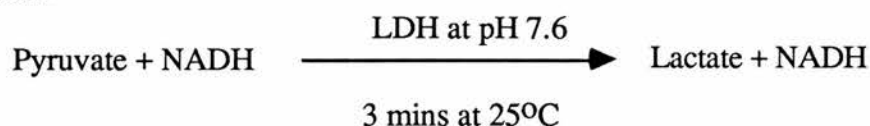
Follow the **decrease** in absorbance at 340nm.

2.5.5. Sample preparation.

Following the appropriate treatment of Sertoli cell or Sertoli cell-germ cell cultures over 24h the culture medium was removed from each well and immediately deproteinized with an equal volume of ice-cold 1.0M perchloric acid. The samples were mixed and centrifuged at 2500g for 10 mins at 4°C. The concentration of lactate and pyruvate were then measured in the decanted supernatants. For each experiment standard solutions of lactate and pyruvate in culture medium were prepared and processed in an identical manner to the samples.

2.5.6. Lactate assay.

The following solutions were added to a 4ml cuvette, mixed and incubated at 25°C for 1h: 2mls 0.5M glycine/0.4M hydrazine buffer pH 9.0, 0.2ml acidified sample or standard, (25mM stock solution diluted with medium to give a range of standards 0-2.5mM), 0.2ml 27mM NAD$^+$ solution and 0.02ml LDH solution (16U/20μl stock diluted 1:12 with 2ml glycine/hydrazine buffer, 0.2ml perchloric acid and 0.2ml enzyme suspension).

The increase in A$_{340nm}$, (as an endpoint of the assay), was measured against a reagent blank, (with 0.2ml perchloric acid replacing the sample), using a Perkin Elmer λ 5UV/vis spectrophotometer. The concentration

of lactate in the samples was calculated by reference to a standard curve of ΔA_{340nm} vs [lactate] mM.

Conditions within the lactate assay are such that the reaction involving LDH favours the production of pyruvate and NADH ie. alkaline pH, excess NAD^+ and hydrazine included in the assay buffer. Hydrazine forms a complex with pyruvate which is not metabolised by LDH (Gutman & Wahlefeld, 1974).

2.5.7. Pyruvate assay.

To 3 mls of acidified sample or standard (100mM stock solution diluted in culture medium to give a range of standards from 0-0.1mM), 1.5mls 0.7M tripotassium phosphate solution was added to neutralise the solution and mixed quickly to prevent localization of alkalinity. After incubating on ice for 15 mins the precipitate (potassium perchlorate) was removed by centrifugation at 2500g for 10 mins at 4°C. The resulting supernatant was assayed for pyruvate. To 0.9mls of the neutralised extract, 1ml 0.5M triethanolamine/0.05M EDTA (pH 7.6) was added, mixed in a cuvette and equilibrated at 25°C. Then 0.08ml NADH (5mg/ml) and 0.02ml LDH solution (6U/20µl stock diluted 1:32 with distilled water) were added to the cuvette and the initial A_{340nm} measured against a buffer blank in a Perkin Elmer λ 5uV/vis spectrophotometer. After a 3min reaction time (endpoint of the reaction) the final A_{340nm} was recorded and the change in A_{340nm} calculated (A_{340nm} final - A_{340nm} initial). The concentration of pyruvate in the samples was calculated by reference to a standard curve of ΔA_{340nm} vs [pyruvate] in mM.

2.5.8. Cell culture protein estimation.

The concentration of protein was determined by the method of Bradford (1976). Following toxicant treatment and the removal of culture medium, 1ml sterile saline was added to each well and sonicated. The content of each well was then stored at -20°C for protein estimation.

Protein concentration per well was measured using a commercially available kit (Protein assay Kit, Biorad, U.K.) using bovine serum albumin (BSA fraction V) as a standard. Results were calculated by log-logit transformation. All protein samples per experiment were assayed simultaneously.

This is a spectrophometric assay in which the absorbance of the solution is measured at a wavelength of 595nm against a saline blank in a Pye-

Unican SP6-500UV spectrophotometer. The concentration of protein was determined from the standard curve of A_{595nm} vs [BSA] (mg/ml).

The loss of protein through germ cell exfoliation due to toxicant treatment was estimated by centrifuging medium, (400g for 5 secs), taken from cultures 24h after toxicant treatment and measuring the precipitate for protein content. Since cells were plated at the same density per well in all experiments and since toxicants in all cases induced exfoliation of germ cells (and so lowered total protein content/well), protein estimations per experiment and between experiments varied. Therefore inhibin, lactate and pyruvate secretion in culture have been expressed per ml of medium, (assuming the same volume/well was used continuously), rather than per mg of protein/well.

2.6. PREPARATION OF SEMINIFEROUS TUBULE CULTURES.

2.6.1. Seminiferous tubule isolation procedure.

Rats (Sprague Dawley, Edinburgh, Scotland) were killed rapidly by inhalation of CO_2 followed by cervical dislocation. An incision was made into the scrotal sac, the testes dissected free of the epididymides and connective tissue, blotted and a small incision made in the caudal end of the testicular capsule. The testes were placed into sterile culture medium M199H containing Hanks salts and 20mM Hepes and stored on ice until required. When necessary a testis was decapsulated and placed into a petri dish containing culture medium M199H at $4^{o}C$ on a cooled stage illuminated from below. Lengths of tubules were then isolated by gently teasing apart the testicular tissue using fine watchmakers' forceps. Care was taken to ensure that tubules were isolated free from interstitial and connective tissue. Tubules were isolated in lengths of >2cm, (approximate range 2-8cm), and these were transferred to fresh medium, (washing step), before being cut with a scalpel to the appropriate size for culture. The cut sections of tubule were then placed into 24-well tissue culture plates on ice, each well containing 100µl of culture medium M199H.

Following the addition of further medium to the appropriate volume and treatment, plates were incubated at $32^{o}C$ in a humified atmosphere of 95%air/5%CO_2. Medium was extracted using a pipette and stored at $-20^{o}C$ for later analysis.

2.6.2. Validation of the seminiferous tubule culture technique.

Inhibin secretion by isolated seminiferous tubules was used as an index of Sertoli cell function. Inhibin secretion was measured using an in-house radioimmunoassay described in Section 2.3.3. In each experiment wells contained medium alone (basal inhibin secretion) or either 2.5 μg/well dibutyryl cyclicAMP (db cAMP) - (Sigma, U.K.) or 0.1 ng/well rat FSH-I-3 (rFSH)-(NIAMDD) (stimulated inhibin secretion). Both stimulants were added at maximally-stimulating concentrations (see below).

The following experiments were performed in order to establish the optimal experimental conditions *in vitro* for seminiferous tubule culture and Sertoli cell inhibin secretion. In each experiment seminiferous tubules from one testis were used, however if more tissue was required seminiferous tubules from the contralateral testis of the same animal were also used to reduce variability of responsiveness. In larger experiments requiring more than one animal, seminiferous tubules from each testis were randomised throughout each well and between culture plates in order to reduce variation of inhibin secretion between testicular tissue taken from different animals.

2.6.2.1. Evaluation of the appropriate culture medium required to maintain seminiferous tubules in culture.

The potential of three different culture media to maintain seminiferous tubules in culture was evaluated using inhibin secretion as an index of function. The composition of each medium was as follows:

1) Basic medium (re Sharpe & Cooper, 1983): Medium M199E containing Earle's salts, 100U penicillin and 100mg streptomycin/ml, 4.0mM L-glutamine and 0.5μg/ml bovine serum albumin (High grade)-(Sigma, U.K).

2) Sertoli cell culture medium (re Williams & Foster, 1988): Eagles MEM containing 0.1mM non-essential amino acids, 4.0mM L-glutamine, 50U penicillin and 50mg streptomycin/ml.

3) Seminiferous tubule culture medium (re Parvinen *et al*, 1980): Hanks F12: Dulbecco's medium (50:50) containing 2.25mM Hepes, 10 mg/ml insulin (Sigma), 3.7×10^{-4} IU/ml ceruloplasmin (Sigma), 5 ng/ml transferrin (Sigma), 2mM L-glutamine and 5 ng/ml gentamycin (Flow Labs).

Tubules were dissected as described in section 2.6.1. Initially, 10cm of tubule/well in individual 2cm lengths were used and each well contained 500μl of the appropriate medium. Inhibin secretion was stimulated by db cAMP (1.0 μg/well) or rat FSH (0.05 ng/well), replacing the appropriate medium and treatment every 24h over a 72h period.

Results.

Cultures maintained in basic (Fig 2.2. a) and Sertoli cell medium (Fig 2.2. b) were significantly more responsive to db cAMP and rat FSH than those in Parvinen medium (Fig 2.2. c) as judged by the magnitude of increase in inhibin secretion. Basic medium sustained consistently higher responses over a wider time period, (i.e. from day 1 onwards), in comparison to cultures maintained in Sertoli cell medium. Basal levels of inhibin secretion for cultures maintained in basic medium and Sertoli cell medium fluctuated slightly but not significantly over the three day culture period. In conclusion, basic medium maintained seminiferous tubules better in culture, (in terms of inhibin secretion), than did Sertoli cell culture medium or Parvinen medium, and therefore was used to culture seminiferous tubules in all further experiments.

2.6.2.2 Evaluation of the concentration of db cAMP and rFSH required to maximally stimulate inhibin secretion.

The potential of a number of doses of db cAMP and rFSH to maximally stimulate and maintain inhibin secretion over a number of days in culture was investigated. Tubules were dissected as previously described (see section 2.6.1.) and 10cms of tubule/well, (in 2cm sections), were cultured in 500μl basic medium. Inhibin secretion was stimulated by varying concentrations of db cAMP or rFSH replacing the appropriate treatment every 24h over a 72h period.

Results.

In culture, db cAMP stimulated inhibin secretion in a dose-dependent manner (Fig 2.3.) in comparison to basal levels up to a dose of 2.5 μg/well but not at 25 μg/well, over the 3 days in culture (Fig 2.3 a). During the third day in culture, the response elicited by db cAMP was reduced significantly at a number of doses but particularly at 25μg/well.

In culture, rFSH also stimulated inhibin secretion in a dose dependent manner in comparison to basal levels up to a dose of 0.1 ng/well but not at 1.0 ng/well (Fig 2.3. b). This pattern was observed over the 3 days in
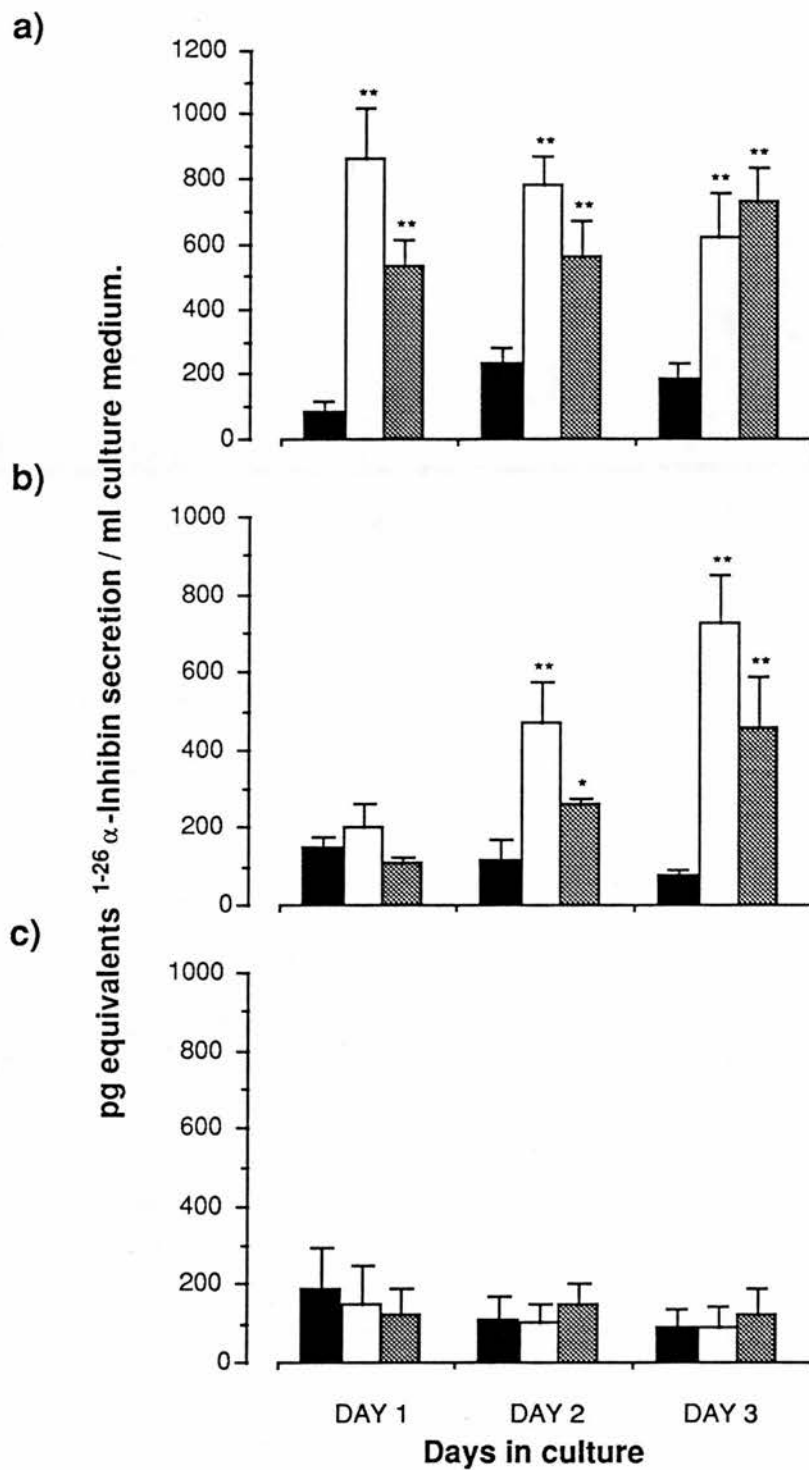
Fig 2.2. Comparison of the effects of three different culture media on
$^{1-26}\alpha$-inhibin secretion by isolated seminiferous tubules using; a) basic medium,
b) Sertoli cell culture medium or c) Parvinen medium, under basal (■) or
stimulated conditions using dibutyryl cAMP (☐) or rat FSH (▨). Values are
means ± SD for four replicates (*P<0.01, **P<0.001, in comparison to basal
secretion on respective days). Results of a representative experiment are
shown.
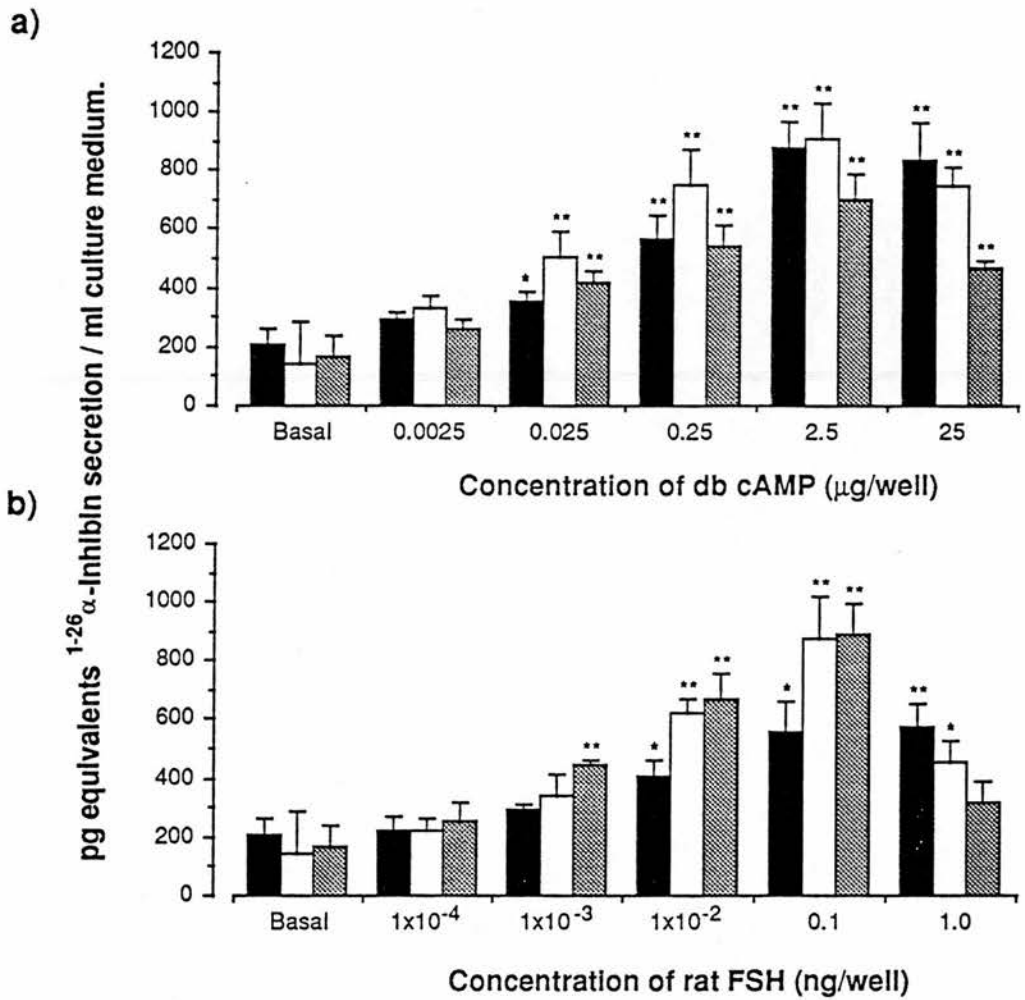
Fig 2.3. Effect of increasing doses of dibutyryl cyclicAMP (a) or rat FSH (b) on $^{1-26}\alpha$-inhibin secretion by isolated seminiferous tubules on day 1 (■), day 2 (☐) or day 3 (▨) of culture. Values are means ± SD for four replicates (*P<0.01, **P<0.001, in comparison to basal secretion on respective days). Results of a representative experiment are shown.

culture, although rFSH elicited a greater response on days 2 and 3 of culture than on day 1. At a dose of 1.0 ng rFSH/well inhibin secretion decreased after 1 day to levels which were not significantly different from basal values on day 3 of culture.

In view of these findings, all subsequent experiments utilized doses of db cAMP and rFSH which consistently stimulated and maintained maximal inhibin secretion over the 3 day culture period. These doses were 2.5 µg db cAMP/well and 0.1 ng rFSH/well.

2.6.2.3. Evaluation of the optimal culture period for seminiferous tubule inhibin secretion and the effect of repeated daily stimulation with rFSH or db cAMP.

The optimal culture period over which inhibin secretion was measured and the effect of continued daily stimulation by rFSH or db cAMP in comparison to a single administration of these stimulants at time 0 was investigated. Tubules were dissected as described in section 2.6.1. and 10cms tubule/well, (in 2cm sections), were cultured in 500µl basic medium. Inhibin secretion was stimulated by db cAMP (2.5 µg/well) or rFSH (0.1 ng/well). Cultures were dosed at time 0 and either terminated at 24, 48, 72, 96, 120 or 148h after culturing or appropriate treatments were replaced every 24h up to 148h in culture.

Results.

Repeated administration of db cAMP to cultures every 24h for up to 3 days stimulated and maintained inhibin secretion to equivalent levels (Fig 2.4. a). From 3 to 6 days a gradual decline in responsiveness was evident. Also during this period an increase in inhibin release under basal conditions was measured such that after 6 days in culture db cAMP stimulated inhibin secretion and basal inhibin release were not significantly different. Levels of inhibin in culture 1 day after the administration of a single dose of db cAMP were similar in magnitude to those measured at day 1 in the repeated administration group (Fig 2.4. b). However during successive days in culture these levels declined in comparison to repeated db cAMP stimulated levels on successive days, whilst basal inhibin secretion increased substantially and was not significantly different from db cAMP stimulated levels after 4, 5 or 6 days in culture.

Repeated administration of rFSH stimulated inhibin secretion in comparison to basal secretion during the first 5 days in culture, reaching a zenith at 3 days (Fig 2.4. c). Due primarily to the rise in basal secretion between 5
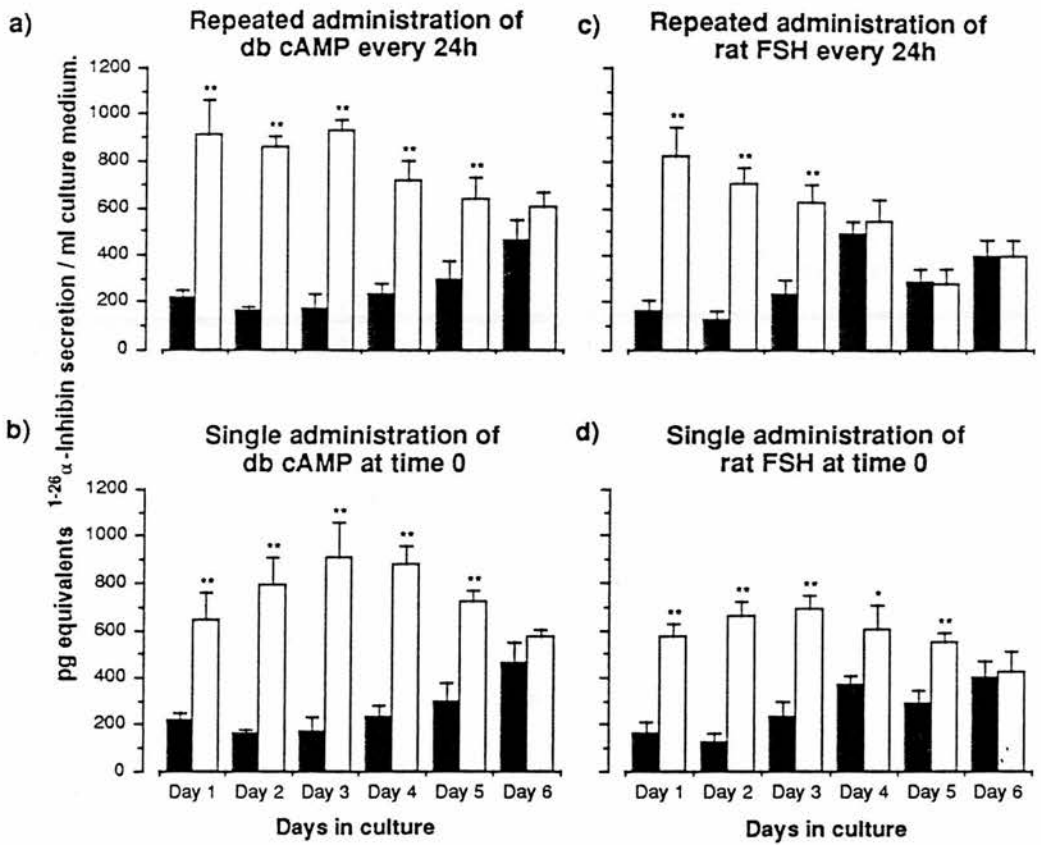
Fig 2.4. Comparison of basal (■) or stimulated [1-26]α-inhibin secretion (☐) using a number of different dosing regimes: Repeated administration of dibutyryl cyclicAMP (a) or rat FSH (c) every 24h, or a single dose of db cyclicAMP (b) or rat FSH (d) administered at time 0 followed by the removal of culture medium after 24, 48, 72, 96, 120 or 144h post-treatment. Values are means ± SD for four replicates (*P<0.01, **P<0.001, in comparison to basal secretion on respective days). Results of a representative experiment are shown.

and 6 days, rFSH-stimulated and basal levels were not significantly different after 6 days in culture. A single administration of rFSH at time 0 stimulated inhibin secretion after 1 day to equivilent levels to those measured after 1 day in the repeated administration group (Fig 2.4. d). However levels did not rise significantly after 2 and 3 days in culture suggesting that repeated administration is required to potentiate rFSH-stimulated inhibin secretion over this period. Furthermore during 4 to 6 days in culture, rFSH-stimulated inhibin secretion declined whilst basal inhibin secretion increased at 4 and 6 days in culture such that by 6 days FSH-stimulated and basal inhibin secretion were not significantly different.

In conclusion, repeated administration of db cAMP and rFSH maintained and potentiated inhibin secretion in comparison to a single administration at time 0. Whilst it is assumed that in single dose experiments the levels of inhibin secretion were equivalent between those measured in day 1 cultures and in the remaining single dose cultures at that time point, the decline in immunoactive inhibin at further time points is suggestive of a progressive breakdown or loss of activity of inhibin during the increased time period in culture. Furthermore, in all experiments during the 4 to 6 day culture period, basal inhibin secretion increased significantly suggesting a fundamental change in the release or secretion of inhibin from the tubules in culture at these times, perhaps reflecting a breakdown in normal physiological processes within the tubule. Therefore in all subsequent experiments db cAMP and rat FSH were administered daily to cultures over a 3 day culture period removing and replacing medium every 24h.

2.6.2.4. Evaluation of the influence of seminiferous tubule quantity per well on inhibin secretion.

The optimal quantity of seminiferous tubule per well required to detect inhibin secretion and the influence of possible factors secreted in relation to the length of tubule cultured and their possible effects on inhibin secretion were investigated. Tubules were dissected as described in section 2.6.1. and cultured in 500µl basic medium. Inhibin secretion was stimulated by db cAMP (2.5 µg/well) or rFSH (0.1 ng/well) replacing the appropriate treatment every 24h over a 72h period. Each well contained either 5,10, 15 or 20cm of seminiferous tubule in 2cm lengths, with the addition of a 1cm length to the 5 and 15cm groups.

Results.

Seminiferous tubule inhibin secretion was significantly stimulated by db cAMP and rFSH in all groups over the 3 days in culture (Fig 2.5). A 2- to 3- fold increase in db cAMP- and rFSH-stimulated inhibin secretion occurred in the 5 and 10cm group after 1, 2 and 3 days in culture (Fig 2.5. a, b and c respectively). The magnitude of this difference however was not consistent between the 10 and 15 and the 15 and 20cm cultures. This was not due to a lack of nutrients in the medium inhibiting inhibin secretion since the pH of the medium, (which gives some indication of metabolic activity), did not change appreciably. After 1 day in culture the levels of inhibin in db cAMP- and rFSH-stimulated cultures were raised significantly in the 15 or 20cm culture groups in comparison to those levels in the 10cm group, however these differences were not observed after 2 or 3 days in culture (Fig 2.5. a, b and c).

Inhibin secretion in the 5cm group was always significantly less than that in the 10, 15 or 20cm groups, independent of treatment. In terms of basal inhibin secretion, there was a direct relationship to the length of tubule in culture. However the difference between basal secretion and db cAMP- or rFSH-stimulated inhibin secretion was always greater in the 10cm group than in the 5, 15 or 20cm group, suggesting that the 10cm group was more responsive to db cAMP and rFSH stimulation than were the others.

In conclusion, there was a direct relationship between the total length of tubule in culture and inhibin secretion in the presence or absence of stimulation. There was also a direct relationship between the duration of culture and the total length of tubule in culture. Therefore, whilst the quantitative secretion of inhibin differed between groups and over the 3 days in culture, the culturing of lengths of tubules up to 20 cms/well represented no practical advantage over those at 10 cm/well, since responsiveness did not increase substantially in the former group. Therefore, 10 cm tubule/well was used in all subsequent experiments.

2.6.2.5. Evaluation of the influence of individual seminiferous tubule length on inhibin secretion in culture.

The effect of individual seminiferous tubule length on basal and stimulated inhibin secretion was investigated in order to identify the optimum length for culture. Tubules were dissected as described in section 2.6.1. and cultured in 500μl basic medium. Inhibin secretion was stimulated by db cAMP (2.5 μg/well) and rFSH (0.1 ng/well) replacing the appropriate treatment every
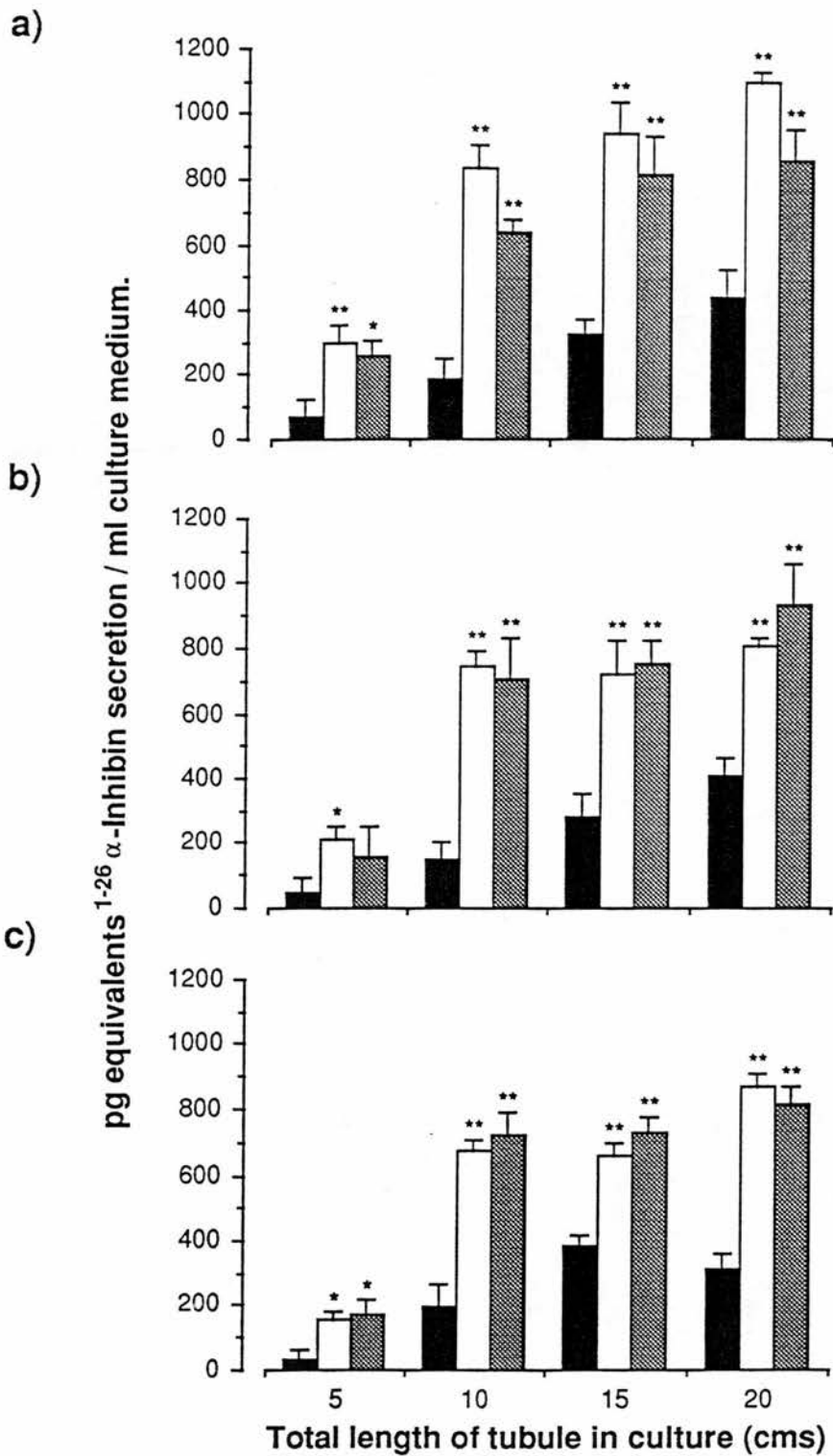
Fig 2.5. Effect of different total lengths of isolated seminiferous tubule on
$^{1\text{-}26}\alpha$-inhibin secretion under basal (■) or stimulated conditions using
dibutyryl cyclicAMP (□) or rat FSH (▨) on day 1 (**a**), 2 (**b**) or 3 (**c**) of
culture. Values are means ± SD for four replicates (*P<0.01, **P<0.001, in
comparison to basal secretion on respective days). Results of a representative
experiment are shown.

24h over a 72h period. Each well contained a constant 10cm of seminiferous tubule ranging in individual lengths thus:- 10 x 1cm, 5 x 2cm or 3 x 3.3cm.

Results.

Inhibin secretion was stimulated by db cAMP and rFSH to equivalent levels in both the 2.0 and 3.3cm tubule groups over the 3 days in culture (Fig 2.6. a, b and c). Basal inhibin secretion also did not vary significantly between these two groups over the culture period. However whilst the levels of inhibin stimulated by db cAMP and rFSH in the 1.0cm tubule culture group were equivalent to those in the 2.0 or 3.3cm culture groups on day 1, these levels declined over the second day and were not significantly different from basal levels following 3 days in culture. This was partially due to a time-dependent decline in responsiveness of the 1.0cm tubules and more significantly due to an increase in basal inhibin secretion by this culture group over the 3 day period. This time-dependent rise in basal inhibin secretion could be due to the leakage of inhibin from within the tubules, or possible due to the leakage of inhibin from cells damaged around the sectioned ends of the tubules. This 'leakage' of inhibin could be an indication of cellular degeneration and therefore, in terms of inhibin secretion, 1.0cm tubule lengths were considered to represent a sub-optimal culture system.

In conclusion, no measurable difference existed between the 2.0 and 3.3cm tubule lengths in culture in terms of basal or stimulated inhibin secretion. However since it is more difficult to mechanically dissect 3.3cm than 2.0cm lengths of tubule, only 2.0cm lengths were used in all subsequent experiments.

2.6.2.6. Evaluation of the influence of culture medium volume per well on inhibin secretion.

The potential of culture medium volume per well to influence basal and stimulated inhibin secretion was investigated in order to identify the optimal volume for culturing seminiferous tubules. Tubules were dissected as described in section 2.6.1. and 10cms tubule/well, (in 2cm sections), were cultured in a range of volumes of basic medium e.g. 250, 500, 750, 1000, 1250 or 1500µl. Inhibin secretion was stimulated by db cAMP (2.5 µg/well) or rFSH (0.1 ng/well), replacing the appropriate treatment every 24h over a 72h period.
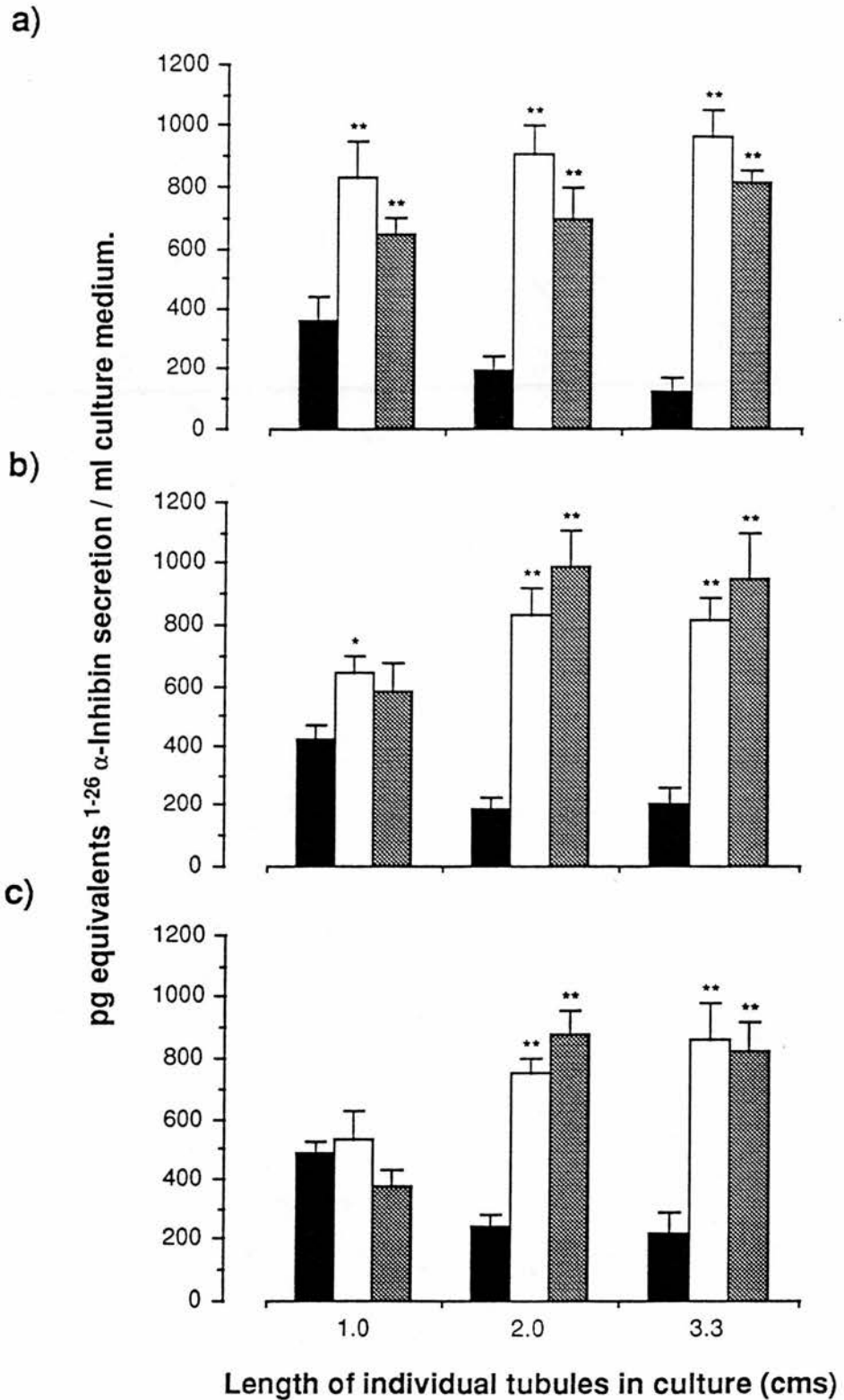
Fig 2.6. Effect of the individual length of isolated seminiferous tubule on $^{1-26}\alpha$-inhibin secretion under basal (■) or stimulated conditions using dibutyryl cyclicAMP (□) or rat FSH (▨) on day 1 (**a**), 2 (**b**) or 3 (**c**) of culture. Values are means ± SD for four replicates (*P<0.01, **P<0.001, in comparison to basal secretion on respective days). Results of a representative experiment are shown.

Results.

Both db cAMP and rFSH stimulated inhibin secretion in a characteristic pattern within individual groups, however the quantitative levels of inhibin varied significantly between groups according to the volume of medium used and the duration in culture (Fig 2.7. a, b and c). In volumes of 250, 500 or 750μl of culture medium db cAMP and, to a limited extent rFSH, stimulated inhibin secretion to significantly higher levels than those in volumes of 1000, 1250 or 1500μl of culture medium following 24h in culture (Fig 2.7a). These differences were also observed on day 2 in culture (Fig 2.7. b) with the exception that the tubules cultured in 250μl of medium secreted significantly less inhibin on day 2 in comparison to the first day, and significantly less than the tubules cultured in 500 and 750μl of culture medium (Fig 2.7. b). Also on the second day of culture, basal inhibin secretion was raised in a number of groups (with the exception of 500 and 750μl) such that by day 3 of culture, (Fig 2.7. c) basal inhibin secretion was equivalent to db cAMP and rFSH stimulated levels in all except the 500 and 750μl groups. Because raised basal levels of inhibin secretion may be representative of 'cellular leakage' rather than secretion, the 250, 1000, 1250 or 1500μl groups of culture medium may represent less physiological culture conditions *in vitro* incapable of sustaining seminiferous tubules over a 3 day period. For practical reasons a volume of 500μl was selected for use in all subsequent experiments.

2.6.2.7. Evaluation of capillary matting as a medium to retain seminiferous tubules in culture.

The potential of an inert medium for retaining seminiferous tubules in culture to reduce or prevent any mechanical damage caused during media removal and replacement was investigated. Tubules were dissected as described in section 2.6.1. and 10cm tubule/well, (in 2cm sections), were cultured in 500μl of basic medium. Inhibin secretion was stimulated by db cAMP (2.5 μg/well) or rFSH (0.1 ng/well) replacing the appropriate treatment every 24h over a 72h period. In each of the treatment groups, direct comparisons between medium and tubules alone or medium and tubules maintained on a 5 x 5mm piece of capillary matting were made.

Results.

No statistically significant differences in basal, db cAMP- or rFSH-stimulated inhibin secretion were observed between tubules cultured in the presence and absence of capillary matting (Fig 2.8. a, b and c). The pattern of
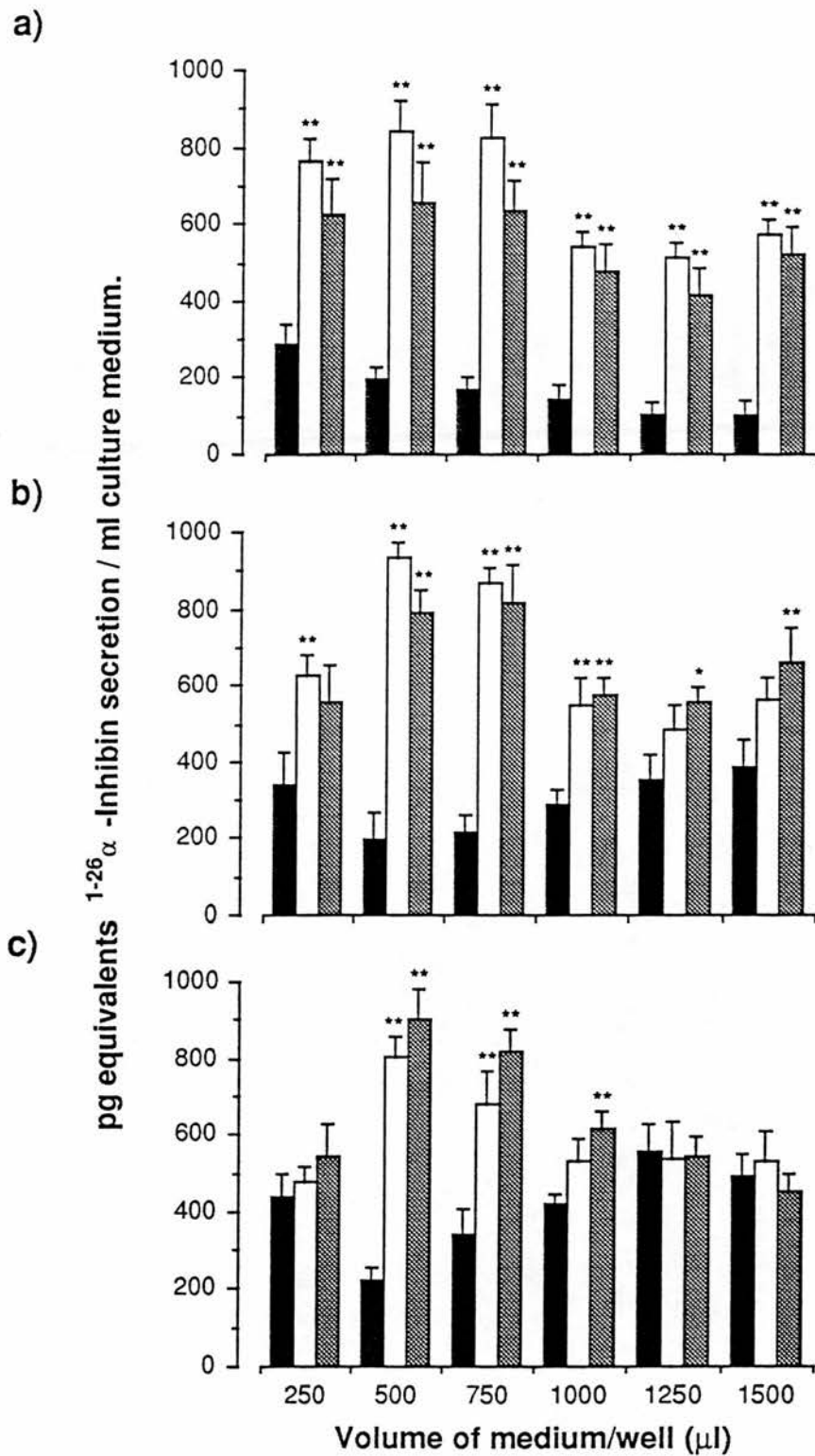
Fig 2.7. Comparison of the effects of culture medium volume on $^{1-26}\alpha$-inhibin secretion by isolated seminiferous tubules under basal (■) or stimulated conditions using 2.5 µg/well dibutyryl cyclicAMP (□) or 0.1ng/well rat FSH (▓) on day 1 (**a**), 2 (**b**) or 3 (**c**) of culture. Values are means ± SD for four replicates (*P<0.01, **P<0.001, in comparison to basal secretion on respective days). Results of a representative experiment are shown.
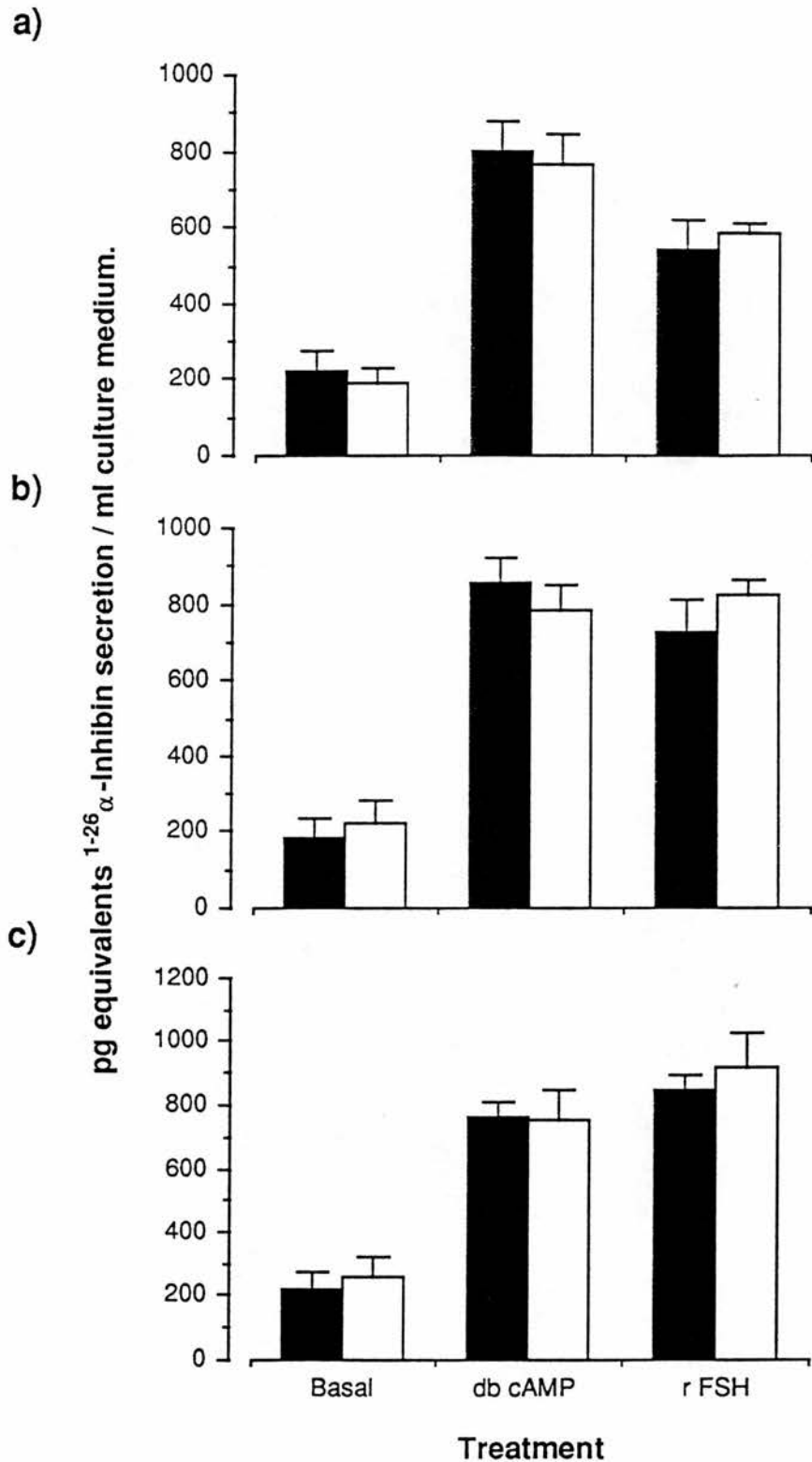
Fig 2.8. Comparison of the effects of capillary matting (□) versus medium alone (■) on $^{1\text{-}26}\alpha$-inhibin secretion by isolated seminiferous tubules under basal, dibutyryl cyclicAMP- or rat FSH-stimulated conditions on day 1 (a), 2 (b) or 3 (c) of culture. Values are means ± SD for four replicates.
No significant differences (P>0.05) were observed. Results of a representative experiment are shown.

secretion in the presence or absence of capillary matting also did not alter on each of the three days in culture. Therefore there were no experimental advantages to culturing seminiferous tubules on capillary matting and, because of technical difficulties which occurred in experiments in which capillary matting was used, all subsequent experiments utilized cultured seminiferous tubules in medium alone.

2.7. Statistics

For hormone measurements, for lactate, pyruvate and inhibin production by Sertoli cell monocultures and co-cultures, and for exfoliation of germ cells in co-culture, Analysis of Variance and Student's t test were performed on the results. These statistical methods were also applied to the differences in basal and stimulated inhibin secretion in cultures of isolated seminiferous tubules. P values of ≤ 0.05 were chosen to represent a statistically significant difference between treatment and control groups. For the evaluation of the change in the frequency of occurrence of stages (see Chapter 4), two-way Analysis of Variance with replication was used.

CHAPTER 3.

META -DINITROBENZENE.

3.1. INTRODUCTION.

Dinitrobenzene (DNB), is an aromatic nitro compound widely used as a chemical intermediate for various organic syntheses, primarily in the production of certain dye intermediates and dyes and in the manufacture of explosives. Secondary to this it is also used to some extent in the plastics production industry. Commercially available DNB is generated by the nitration of nitrobenzene, the result consists of a mixture of the *ortho, meta* and *para* isomers of nitro group ring substitutions. In common with many other nitro aromatic compounds, exposure to the *meta* or *para* isomers can induce methaemaglobinaemia in both experimental animals (Facchini & Griffiths, 1981; Blackburn *et al,* 1988), and in man (Beritix, 1956), and on prolonged exposure results in anaemia (Watanabe *et al*, 1976). The most frequently reported consequence of exposure in man is hypoxia due to methaemaglobinaemia (Beard *et al*, 1981).

Of the three isomers, *meta* (or 1,3) DNB, (mDNB), a yellow crystalline substance at room temperature, is the most economically important isomer and one of the most widely used chemical intermediates in industry in the synthesis of a large number of commercial organic compounds. mDNB is also the most highly toxic to humans. This is because it is readily absorbed through intact skin (Ishihara, 1976), and is also quickly absorbed through the lungs into the blood. mDNB is the most potent of the three isomers in producing methaemaglobin, the principle symptom and predominant toxicological response of acute mDNB intoxication in both man and animals being cyanosis and tissue hypoxia. The probable lethal dose in man is in the range of 5 to 50 mg/kg bodyweight (Gosselin *et al*, 1984), and in rats, the oral LD_{50} is 83 mg/kg bodyweight (Cody *et al*, 1981). Recently, investigations into the toxicity of mDNB in animals have concentrated on its effects on reproductive performance, which is also a probable area of toxicity.

Studies concerned with the potential testicular toxicity of mDNB in animals were first instigated by Cody and co-workers in 1981. They were the first to demonstrate the potential of mDNB in producing testicular atrophy in rats. Subchronic exposure of rats to mDNB administered at a dose of roughly 20 mg/l, (2.6 to 13 mg/kg/d), for 16 weeks in drinking water produced a significant reduction in testes weight, characterised by severe disruption of spermatogenesis and moderate reductions in haemocrit and haemaglobin values. However using this route of exposure it is difficult to accurately evaluate the dose each animal received or to equate the dose ingested to the degree of testicular disruption produced. Further investigations were required to establish; a) the minimum

effective dose resulting in testicular disruption, b) whether the parent compound or a metabolite was the toxicant, c) the initial target site and any possible stage specificity induced by the compound and d) the mechanism of toxicant action. Several studies have addressed these questions employing histological observations after treatment *in vivo* and Sertoli-germ cell co-culture data generated *in vitro*.

Histological investigations into the testicular target site of mDNB were undertaken initially by Blackburn *et al* (1985) and Hess *et al* (1985). This preliminary work indicated that the compound produced time and dose-dependent decreases in testes weight with testicular effects observed at the histological level in rats within 24 hours of a single oral dose of 15 or 25 mg/kg, the initial lesion occurring within the Sertoli cell and resulting in disruption of the seminiferous epithelium and accompanying spermatocyte degeneration. Further histological evaluations using electron microscopy (Foster *et al*, 1986) to observe the seminiferous epithelium after oral administration of mDNB to rats illustrated Sertoli cell cytoplasmic vacuolation initially particularly at stages IX and X of the spermatogenic cycle. These changes were independent to, and before, other disruption of the epithelium and followed by germ cell necrosis suggesting that the Sertoli cell was the initial target of mDNB in producing subsequent stage-specific spermatogenic disruption (Foster *et al*, 1986).

Of the three isomers of DNB, attention has been focussed primarily on mDNB. Overwhelming evidence points towards mDNB as being a testicular toxicant *in vivo* and *in vitro*, with a direct initial effect on the Sertoli cell resulting in disruption of spermatogenesis. However, DNB has another two isomers, *ortho* and *para*. Investigations into the ability of these two isomers to induce testicular degeneration in rats after oral administration were undertaken by Blackburn *et al*, (1988). Whilst both *meta* - and *para* -DNB induced cyanosis and splenic enlargement in rats when administered at identical doses, only *meta* - DNB induced significant decreases in testicular weight and histological changes in the seminiferous epithelium. These effects were dose-dependent being observed at doses ranging from 50 to 200 mg/kg. Using electron microscopy the first observable change in structure (vacuolation) was observed within the Sertoli cell suggesting again that the Sertoli cell was the primary target for the toxic actions of mDNB in the testis, with germ cell damage occurring as a secondary event.

The conclusion drawn from these histological studies is that the testicular atrophy induced by mDNB can be attributed to a direct effect upon the seminiferous epithelium, probably initially via a direct perturbation of Sertoli cell

function. Although histological evidence suggests that spermatogenesis is disrupted in possibly a reversible manner following the administration of a single dose of mDNB to rats it is difficult to acertain the degree of disruption and rate of reversibility per animal using histological examination alone. Non-invasive sequential monitoring of mDNB-induced testicular disruption *in vivo* is therefore difficult since there are no biochemical markers of normal testicular function which may be isolated from readily available sources e.g. blood. The monitoring of sperm output at sequential time intervals after toxicant administration *in vivo*, although not ideal, allows investigators to follow retrospectively the results of testicular lesions and because of the precise chronological nature of spermatogenesis, it is possible to postulate which cell types were initially affected after mDNB-induced Sertoli cell perturbation. Linder *et al* (1986) exposed rats to subchronic oral administrations of mDNB at doses of 1.5 mg/kg/d and induced a decrease in sperm production and, at 3 mg/kg/d rendered male rats infertile. Further experiments were performed by Linder and co-workers (1988) using sperm quality, quantity and fertilising capacity as markers of Sertoli cell perturbation following acute oral administration of mDNB (48 mg/kg-maximum non-lethal dose) to rats. Testicular and epididymal parameters measured at successive time intervals after treatment with mDNB indicated that the compound produced a rapid decrease in testes weight and resulted in hypospermia, poor sperm quality and loss of fertilising capacity post-treatment. Substantial recovery occurred in some animals, but in others little or no remission of testicular damage was observed. Further studies by Foster *et al* (1989) have demonstrated that in mating trials a significant reduction in the percentage of females pregnant after mating to males exposed to mDNB at concentrations of 5 and 10 mg/kg/day for 5 days. Complete recovery was evident in both groups by 63 days after the cessation of treatment.

Once the initial target cell had been identified histologically, the capability of using an *in vitro* system to study Sertoli cell perturbation induced by mDNB was first adopted by Foster *et al* (1986). Initial investigations centred on identifying if the parent compound or a metabolite was distributed within the testis and, if mDNB was the toxicant, identifying the minimum testicular concentration of mDNB required to produce spermatogenic disruption *in vivo*. This and lower concentrations were then used *in vitro* to assess possible Sertoli cell perturbation. In Sertoli-germ cell co-cultures and Sertoli cell mono-cultures, the Sertoli cell monolayer remained intact and confluent, however vacuolation and phagocytosis of degenerating spermatocytes were observed following

mDNB treatment at concentrations relevant to those evident *in vivo*. Dose-dependent exfoliation of essentially viable germ cells of all types from the Sertoli cell monolayer was also evident (Foster *et al*, 1986). Once a morphological response had been established *in vitro*, further investigations were conducted on the metabolism of mDNB *in vitro*. Using ^{14}C labelled DNB, culture media samples were analysed 24 h after exposure to mDNB for metabolic composition using a thin layer chromatographic separation technique. Although only 10 percent of the applied dose was metabolised, the appearance of metabolites was observed to vary with dose and time. The major metabolites detected were *m*-nitroacetanilide and *m*-nitroaniline, neither of which showed any evidence of toxicity when administered to cultures at relevant concentrations (Foster *et al*, 1986). Further metabolite studies conducted *in vitro* by Foster *et al* (1987), again using ^{14}C labelled mDNB, postulated a possible scheme of metabolism within the Sertoli cell. Both Sertoli-germ cell co-cultures and Sertoli cell cultures were capable of xenobiotic metabolism, with nitro reduction of mDNB being the most prominent route. It was therefore postulated that mDNB or a Sertoli cell metabolite (probably an intermediate of nitroreduction produced *in situ*) was responsible for testicular damage observed following administration of this compound *in vivo* (see Foster *et al*, 1989 for review).

The identification of the toxicity of mDNB *in vitro* using histological techniques has allowed observation of the direct or indirect effects of cellular perturbation. However, other than using germ cell exfoliation as a crude index of toxicity, no biochemical index of testicular competence or measure of Sertoli cell perturbation *in vivo* has been identified. Williams (1987) and Williams & Foster (1986; 1988) found that mDNB perturbed normal Sertoli cell biochemical function *in vitro* as deduced from the measurement of two Sertoli cell products, lactate and pyruvate (Jutte *et al*, 1981), which are known to be required for normal germ cell function (Jutte *et al*, 1983). They observed significant, dose-dependent increases in the concentrations of both lactate and pyruvate in the culture medium following mDNB administration, with a decrease in the lactate:pyruvate ratio from control values (10:1). These decreases clearly reflected alterations in Sertoli cell function and were different from those produced by other toxicants e.g. mono-2-(ethylhexyl) phthalate (MEHP), possibly reflecting the differences in aetiology of Sertoli cell damage induced by these compounds *in vivo*. Thus lactate and pyruvate production by rat Sertoli cell cultures may be a sensitve index of altered function and/or dysfunction induced by mDNB. Concomitant studies to those of Blackburn *et al* (1988) but using Sertoli-germ cell co-cultures and Sertoli cell cultures have also

demonstrated isomer specificity *in vitro* (Williams & Foster, 1988). Whilst mDNB induced significant increases in the production of lactate and pyruvate after 24h in culture at relevant testicular concentrations, *ortho* - and *para* - DNB, at identical concentrations, did not alter the production of lactate and pyruvate from that of control levels.

It has therefore been established that the rat is a sensitive species to orally administered mDNB. Such treatments elicited a decrease in testicular weight as a result of a rapid dose-related and stage-specific lesion with the initial changes occuring within the Sertoli cell. The resultant atrophy of the seminiferous epithelium and perturbation (vacuolation) of the Sertoli cell was initially apparent at stages VII to XIII of the spermatogenic cycle, resulting in a time-dependent decrease in fertility of treated rats appearing a number of days after mDNB treatment. Results from fertility trials suggest that the testicular effects induced by mDNB are reversible (Foster, 1989). Further evidence produced by Foster *et al* (1987) suggests that mDNB also produces testicular toxicity *in vitro* in a Sertoli cell or Sertoli-germ cell co-culture system with the Sertoli cell as the probable testicular target for the toxic action of mDNB and that this *in vitro* system effectively models the morphological changes observed with the compound *in vivo* and is capable of xenobiotic metabolism *in vitro*, with mDNB, or a metabolite, (probably a reactive intermediate of nitro reduction produced *in situ*) being responsible for the observed testicular damage *in vitro*. Also the responses evident *in vitro* occurred at and below concentrations equivalent to those observed in blood and testis after a single oral dose of mDNB *in vivo*. From the results obtained *in vitro* for mDNB, the Sertoli cell secretory products lactate and pyruvate may therefore be used as indices of altered biochemical function *in vitro*. Finally, isomer-specific differences do exists *in vivo* and *in vitro* with only *meta* - DNB (as opposed to *ortho* - and *para* - DNB) possessing testicular toxicity.

Although these studies have gone a long way towards addressing the testicular toxic actions of mDNB a number of questions await answers. As the Sertoli cell has important endocrine functions any toxicant which perturbs the cell in a structural manner may also affect its cellular function and will therefore enhance or impair the hormonal secretory function of the Sertoli cells and, as such, may alter the secretory patterns of hormones released, possibly those involved in the hypothalamic-pituitary gonadal axis which controls testicular function. Measurement of hormonal status in a temporal manner following a single oral administration of mDNB may aid in our understanding of the physiological responses involved in spermatogenic regeneration and possibly act

as a marker of dysfunction. The aim of the present investigation was to follow the testicular lesions induced in rats by a single oral dose of mDNB through at least 5 complete spermatogenic cycles, examining temporal changes in the hormonal milieu coupled with histological examination at specific time points post-treatment. Studies *in vitro* using Sertoli cell cultures concentrated on the production of inhibin, a glycoprotein hormone produced by the Sertoli cell in response to FSH, to observe if mDNB had any effect on inhibin production and/or release *in vitro*. Furthermore, the possiblity that inhibin could be utilised as a potential biochemical marker of testicular dysfunction or Sertoli cell competence *in vivo* and *in vitro* was investigated.

3.2. METHODS.

3.2. Methods for assessment of the effects of mDNB *in vivo*.

3.2.1. Reagents.

mDNB was obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and was recrystallised in ethanol to greater than 99% purity as determined by gas chromatography/mass spectrometry. The vehicle for mDNB administration was polyethylene glycol 600 (PEG 600) obtained from Sigma (Poole, Dorset, U.K).

3.2.2. Animals.

Rats were of the Sprague Dawley derived strain bred in the Centre for Reproductive Biology in Edinburgh, U.K.

3.2.3. Experimental Protocol.

Adult male rats (100-140 days old) were randomly allocated 8/cage and maintained under normal animal house conditions. Food and water were available *ad libitum*.

At the beginning of the experiment (time 0) rats were administered mDNB by oral gavage at a single dose of 25mg/kg bodyweight in PEG 600 (1ml/kg). Animals were weighed daily for the first 14 days post-treatment in order to detect any significant decreases in body weight which may have indicated overt toxicity. At selected time intervals after dosing rats were subjected to one of the following procedures;

a) 2 to 3 rats/group were anaesthetised and perfusion-fixed for histological analysis (see section 2.4.1.).

b) 5 to 6 rats/group were killed by CO_2 inhalation followed by cervical dislocation. The testes were removed and weighed, blood was collected immediately after death for hormone measurement (see section 2.3.) and testicular interstitial fluid (IF) was drip collected overnight (see section 2.2.1.) also for hormone measurements. Serum hormones measured were FSH, LH and testosterone whilst the hormones measured in IF were testosterone and inhibin.

The successive time intervals at which groups of mDNB-treated animals were killed for the procedures outlined above were 1, 3, 7, 14, 21, 28, 42 or 70 days post-treatment while control groups were killed at 7, 28, or 70 days post-treatment.

3.3. Methods for assessment of the effects of mDNB *in vitro*.

3.3.1. Reagents.

For the purity of mDNB see section 3.2.1. All culture materials were obtained from Flow Laboratories (Irvine, Scotland, U.K.) or Gibco (Paisley, Scotland, U.K). All biochemical reagents were obtained from Sigma, B.D.H. (Poole, Dorset, U.K.) or Boehringer Mannheim (Lewes, East Sussex, U.K.) and were of the highest purity available.

3.3.2. Animals.

Rats were of the Alpk:AP (Wistar derived) strain from the I.C.I. Animal Breeding Unit, Alderley Park, Macclesfield, Cheshire, U.K.

3.3.3. Experimental Protocol.

3.3.3.1. Preparation of Testicular cell cultures.

Co-cultures were perpared using a modification of the method of Gray & Beamand (1984). Sertoli cell only cultures were prepared from co-cultures using the method of Galdieri *et al* (1981) (see section 2.5.1.).

3.3.3.2. Treatment of Cultures.

Toxicants were administered to cultures 72h after plating. mDNB was administered at a final concentration of between 5×10^{-8} and 5×10^{-3}M over a 24h incubation period and applied as solutions in dimethylsulphoxide (DMSO), final concentration 0.3% v/v, while control cultures received an equal volume of this vehicle. Treatments caused no appreciable alteration of culture

medium pH. All experiments were performed at least three times, in each case with a minimum of six replicates for each dose and treatment group.

3.3.3.3. Parameters measured.

Medium was removed from cultures 24h after toxicant administration for measurement of inhibin (see section 2.3.3.) and germ cell exfoliation (see section 2.5.3.). The remaining cells in each well were used for protein estimation (see section 2.5.8.). Cultures grown on cover slips were fixed and stained using haematoxylin and Papanacolaou's stain for histological examination (see section 2.5.2.).

3.4. RESULTS.

3.4.1. Effect of mDNB on testicular weight.

Treatment of animals with a single oral dose of mDNB induced a time-related decrease in testicular weight over the first 14 days post-treatment (Fig 3.1). At 21, 28 and 42 days post-treatment testicular weight had increased but remained significantly less than the average testicular weight of controls (made up of the sum of the three control groups). By 70 days after treatment with mDNB, the testicular weight had recovered to within the control range (Fig 3.1).

3.4.2. Effect of mDNB on the serum levels of FSH.

Within 24h of a single oral administration of mDNB, serum FSH levels were raised above the average control level and were significantly elevated by 72h post-treatment. Indeed over the first 7 days there was a time-dependent increase in the levels of serum FSH, reaching a zenith at 7 days post-treatment (Fig 3.2). Serum FSH levels remained elevated to an equivalent extent at 14 days post-mDNB treatment. By 21 days post-treatment levels were not significantly different from an average control FSH level made up of the sum of the three control groups. However at 28, 42 and 70 days post-treatment, levels of serum FSH in mDNB-treated rats were again significantly elevated (Fig 3.2).

3.4.3. Effect of mDNB on the serum levels of LH.

In the groups of animals treated with mDNB the levels of serum LH fluctuated markedly between individual rats. Serum LH levels increased progressively between 3 and 14 days after treatment with mDNB. A time-dependent increase in serum LH levels was observed in mDNB-treated rats at

Fig 3.1. Effect of a single oral dose of *meta* dinitrobenzene (25 mg/kg bodyweight) (■) or vehicle alone (1ml/kg) (▦) on testicular weight in groups of rats at intervals after dosing. Values are means ± SD for 6 rats (*P<0.01, **P<0.001, in comparison to an overall control value [dotted line] made up of the sum of the three control groups shown).
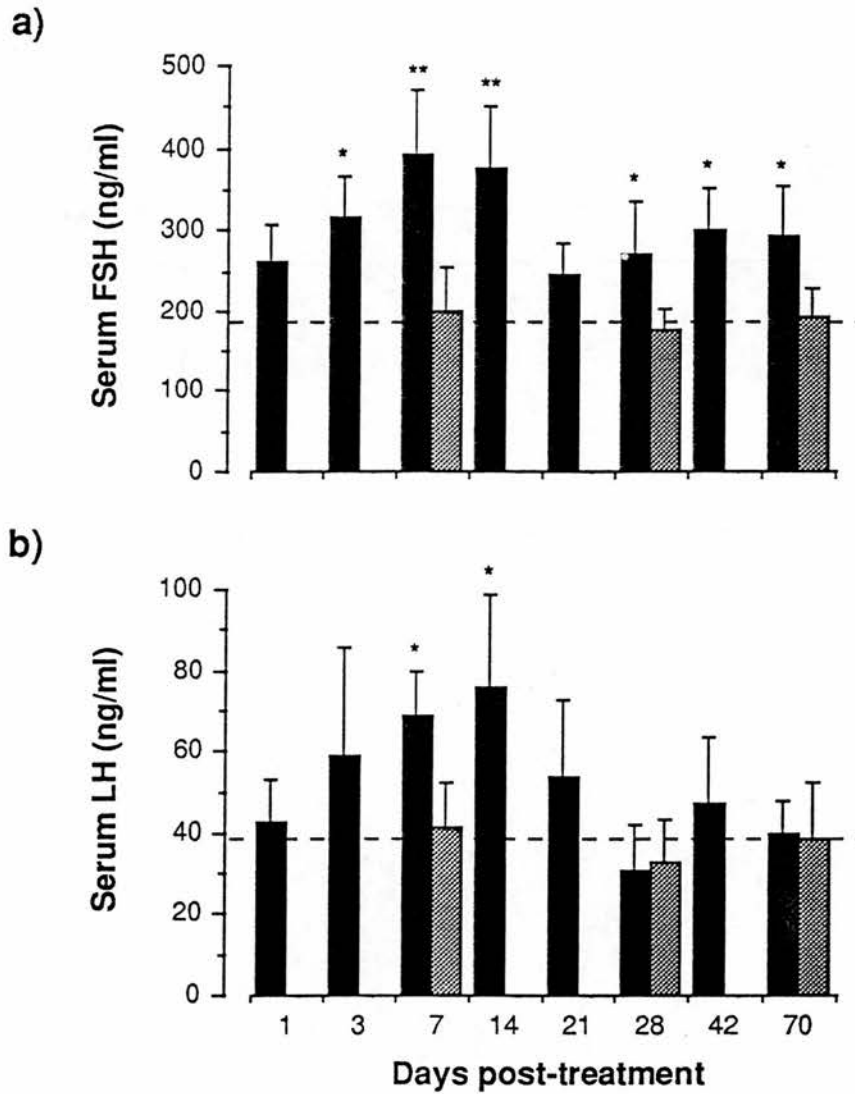
Fig 3.2. Effect of a single oral dose of *meta* dinitrobenzene (25 mg/kg bodyweight) (■) or vehicle alone (1ml/kg) (▨) on the serum levels of FSH (**a**) or LH (**b**) in groups of rats at intervals after dosing. Values are means ± SD for 6 rats (*P<0.01, **P<0.001, in comparison to an overall control value [dotted line] made up of the sum of the three control groups shown).

3, 7 and 14 days post-treatment; serum LH levels at the latter two time intervals were significantly increased in comparison to an average control LH level made up of the sum of the three control groups (Fig 3.2). By 21 days and at 28, 42 and 70 days post-treatment serum LH levels in mDNB-treated rats had declined such that they were not significantly different from the grouped control value (Fig 3.2).

3.4.4. Effect of mDNB on interstitial fluid (IF) levels of inhibin.

The levels of immunoactive inhibin in IF were significantly elevated at 1, 3 and 28 days after mDNB administration, in comparison to an average control level of inhibin made up of the sum of the three control groups (Fig 3.3). The largest increase in IF levels of inhibin was observed 24h after treatment.

3.4.5. Effect of mDNB on the serum and IF levels of testosterone.

The levels of testosterone measured in serum and IF following mDNB administration followed a similar temporal pattern. Within 24h of a single oral dose of mDNB the levels of serum and IF testosterone were increased significantly (Fig 3.4), while levels for the latter were also significantly increased, but to a lesser extent, 3 days post-treatment in comparison with a grouped control level (Fig 3.4).

By 21 and 28 days post-treatment, levels of testosterone in IF were increased significantly but to a smaller degree than at 1 and 3 days post-treatment. Although serum testosterone levels were also raised at 21 and 28 days post-treatment, only the level at the latter time interval was significantly different from the grouped control value (Fig 3.4). At all other time intervals the levels of serum and IF testosterone were not significantly different from the grouped control value.

3.4.6. Effect of mDNB on testicular morphology.

Testicular tissue from control rats in the 7, 28 and 70 day groups exhibited normal histology with no abnormal cellular necrosis or germ cell exfoliation (plates 3.1 a,b and c), and with apparently normal kinetics of spermatogenesis as judged by the frequency of the different stages (Leblond & Clermont, 1952).

Acute exposure of rats to oral mDNB administration caused very rapid and extensive disruption of spermatogenesis in a number of treated animals. However, there was a marked variability between animals in the
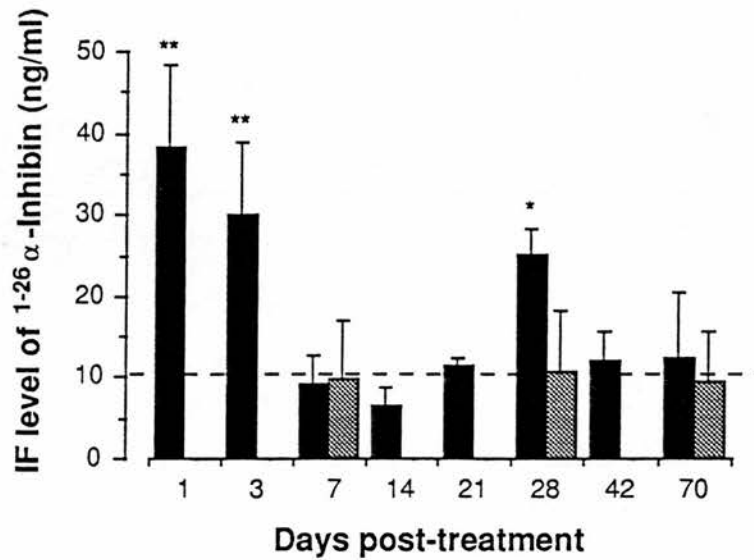
Fig 3.3. Effect of a single oral dose of *meta* dinitrobenzene (25 mg/kg bodyweight) (■) or vehicle alone (1ml/kg) (▓) on levels of immunoactive inhibin in testicular interstitial fluid (IF) from groups of rats at intervals after dosing. Values are means ± SD for 6 rats (*P<0.01, **P<0.001, in comparison to an overall control value [dotted line] made up of the sum of the three control groups shown).
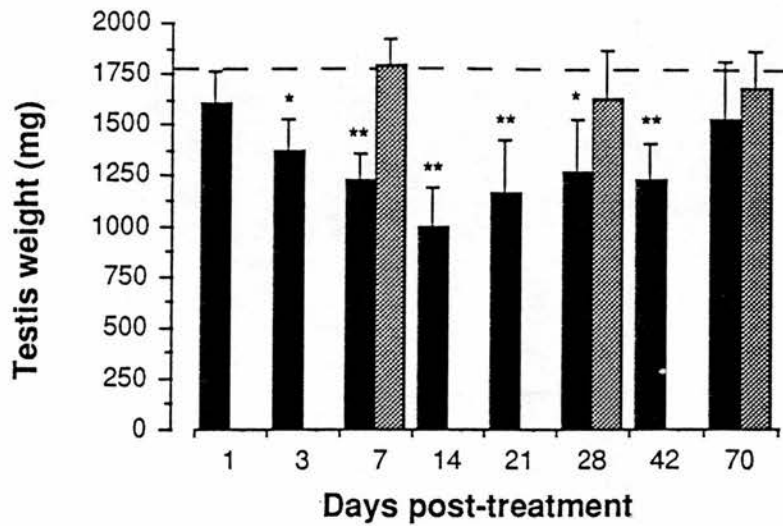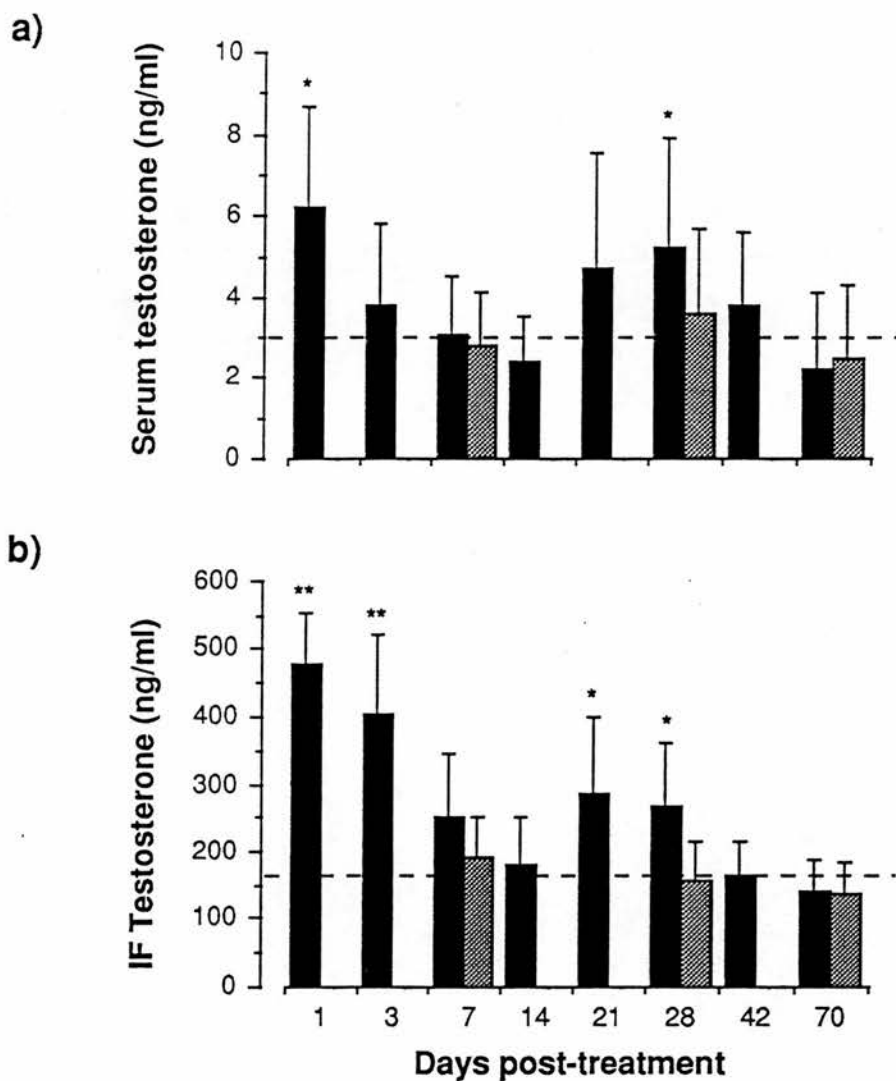
Fig 3.4. Effect of a single oral dose of *meta* dinitrobenzene (25 mg/kg bodyweight) (■) or vehicle alone (1ml/kg) (▒) on the levels of testosterone in serum (**a**) and testicular interstitial fluid (IF) (**b**) in groups of rats at intervals after dosing. Values are means ± SD for 6 rats (*P<0.01, **P<0.001, in comparison to an overall control value [dotted line] made up of the sum of the three control groups shown).
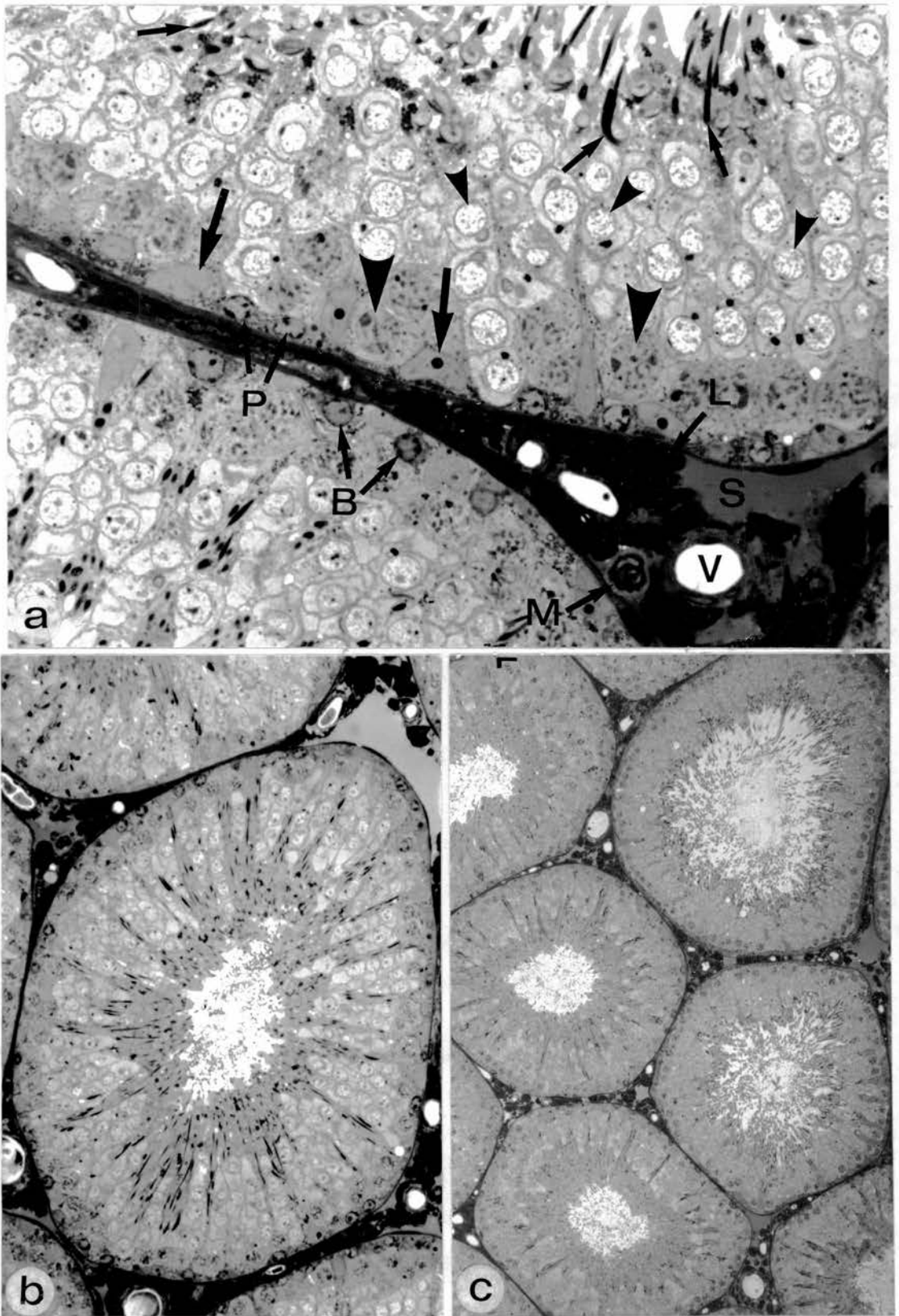
Plate 3.1. Seminiferous tubules from control rats illustrating (a) high power view of epithelium showing
Sertoli cell nuclei (large arrows) pachytene spermatocytes (large arrowheads) round spermatids (small
arrowheads) and the heads of elongate spermatids (small arrows) in a stage VII tubule. Compare also the
preleptene spermatocytes (P) and B spermatogonia (B). The interstitial space (S), blood vessels (V),
Leydig cells (L) and macrophage (M) are also shown (x400); (b) section through a stage IV-V tubule
(x190); (c) low power view through a number of tubules (x75).

degree of testicular disruption, with some animals having extensive tubular dysfunction, with germ cell degeneration and a complete breakdown of spermatogenesis in most tubules, while in other treated animals only the occasional degenerating pachytene spermatocytes and minor Sertoli cell vacuolation were observed with a minimum of disruption to the epithelium.

The most obvious effects of mDNB on the testis at 24h post-treatment were the degeneration of pachytene spermatocytes (plate 3.2 a and b), the vacuolation of Sertoli cells (plate 3.2 c) and the general disorganisation of the seminiferous epithelium (plate 3.2 d). This degeneration was stage-dependent, occurring in tubules in stages VII to XII of the spermatogenic cycle (plate 3.3 a). The darkly staining pachytene spermatocytes (plates 3.2 a and b) were either phagocytosed by Sertoli cells, sloughed into the lumen or formed multinucleated giant bodies as observed at 72h post-treatment. Vacuolation within the epithelium was also observed 24h post-treatment (plate 3.2 c). These vacuoles were not artifacts as they were not accompanied by shrinkage of the tubule around the local area and were not observed in identically processed control sections. These holes therefore represent the absence of developing germ cells probably lost as a result of mDNB-induced degeneration. In contrast, sections taken from the testis of other rats in the same group treated with mDNB showed minimal cellular degeneration and disruption of spermatogenesis. In these animals, only a small number of pachytene spermatocytes appeared pyknotic, and disruption of spermatogenesis was significantly less than that observed in the more affected animals (plate 3.3 b).

By 3 days post-treatment the extent of testicular damage had increased compared to that observed at 24h post-treatment. Irrespective of the spermatogenic stage all pachytene spermatocytes either appeared pyknotic and irregular in shape or had degenerated and were completely absent from the epithelium of tubules (plate 3.3 c). In addition, round spermatids also appeared morphologically different from respective controls, having an opaque appearance to their cytoplasm and containing fragmented chromatin within the nucleus (plate 3.4 c). Overall, the seminiferous epithelium was severely disrupted (plate 3.4 a) and tubules contained large multinucleated giant cells (plates 3.4 b and 3.5 a). These giant bodies appeared to consist of either degenerating pachytene spermatocytes or round spermatids but in no instances were round spermatids and pachytene spermatocytes observed within the same giant body structure (plate 3.5 b and c). Again in contrast to the severity of the effect of mDNB on testicular tissue observed in some rats, in others, mDNB had only minor effects with only minor vacuolation of the Sertoli cells and/or
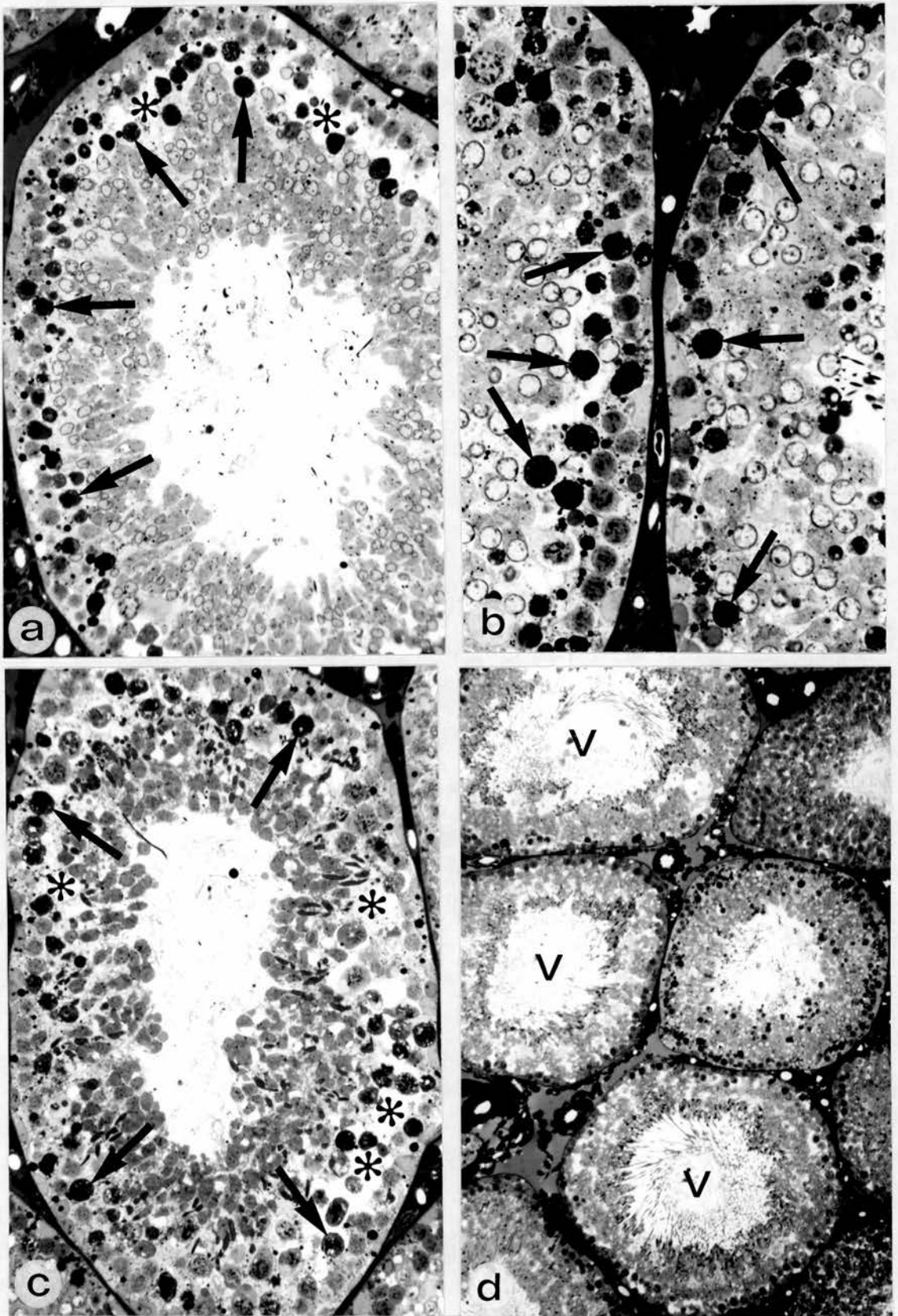
Plate 3.2. Testicular morphology 24h after a single oral dose of mDNB showing (a) degenerating pachytene spermatocytes (arrows) and vacuolation of the epithelium (astericks) (x210); (b) section through two adjacent stage VIII seminiferous tubules illustrating degenerating pachytene spermatocytes (arrows) (x300); (c) stage XII tubule with degenerating pachytene spermatocytes (arrows) in addition to a severely disrupted and vacuolated epithelium (astericks) (x230); (d) lower power view of seminiferous tubules illustrating vacuolation of the epithelium at different stages of the spermatogenic cycle (x75).
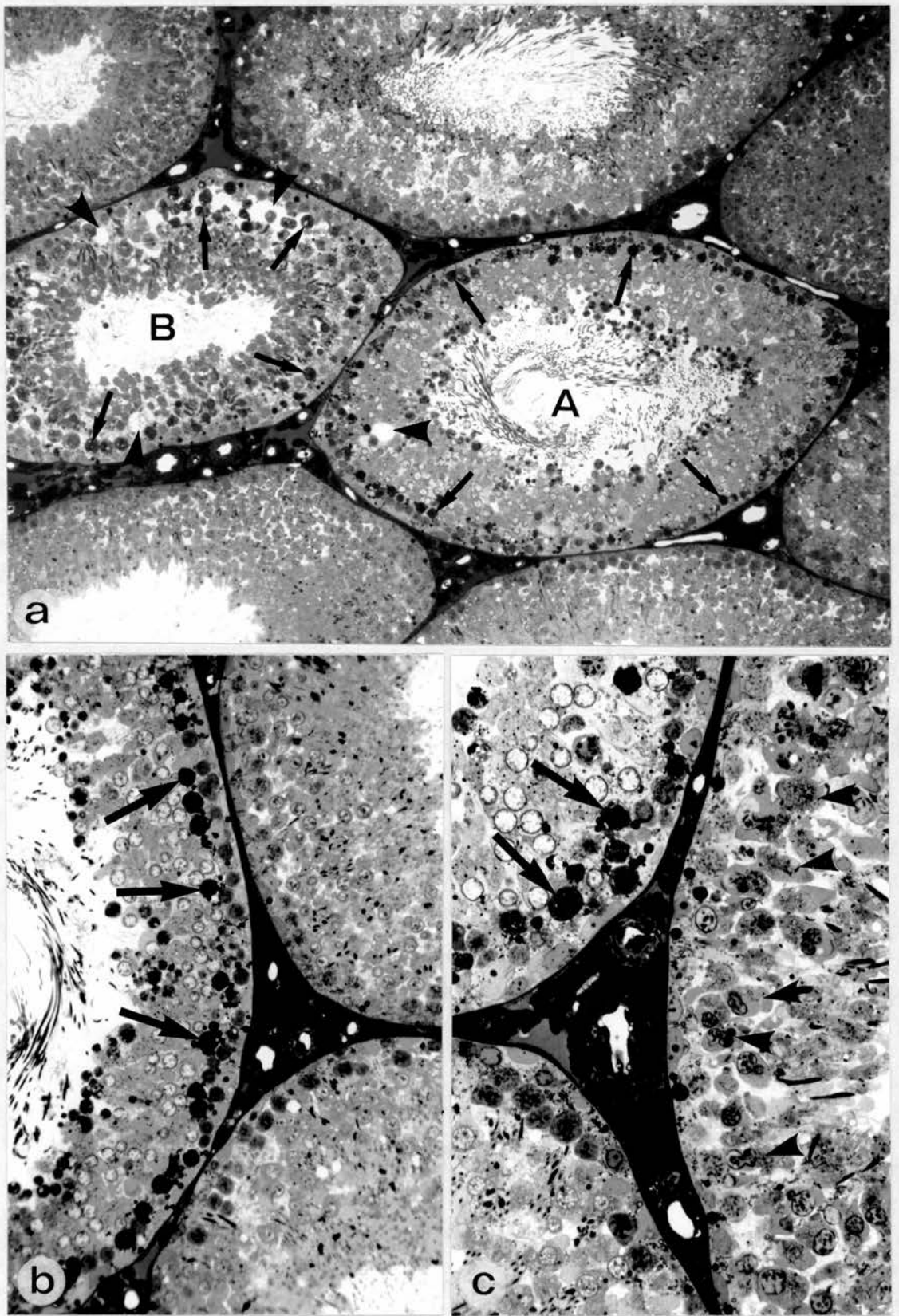
Plate 3.3. Testicular morphology 24h after a single oral dose of mDNB illustrating (a) low power view showing degeneration of pachytene spermatocytes (arrows) and vacuolation of the epithelium (arrowheads) of tubules in stages VIII (A) and XII (B) (x100); (b) section through three adjacent seminiferous tubules showing degeneration of pachytene spermatocytes in a stage VIII tubules (left) (x190); (c) testicular morphology 72h after a single oral dose of mDNB illustrating degenerating pachytene spermatocytes (arrows) together with round spermatids which appear irregular in shape (arrowheads) (x300).

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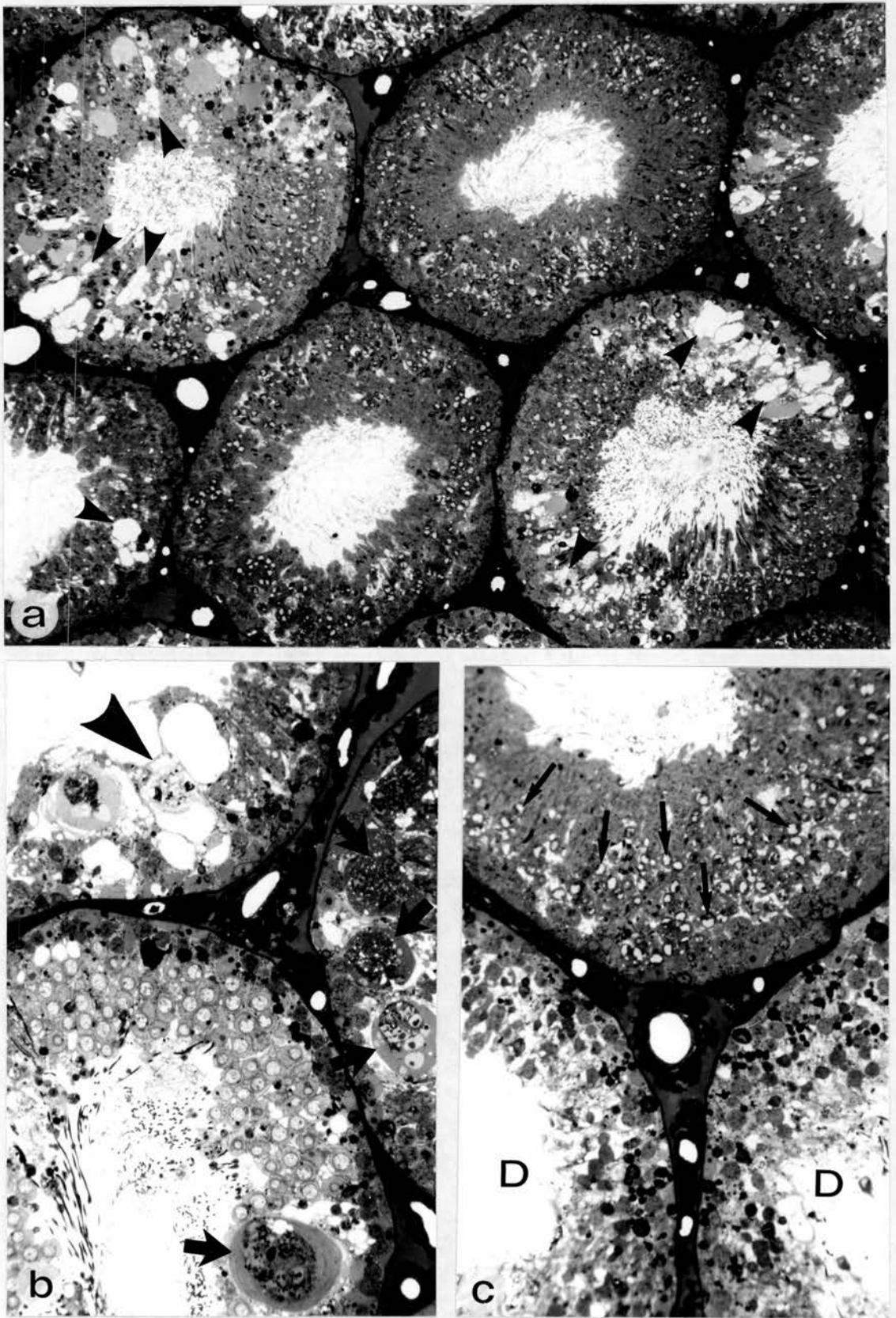
Plate 3.4. Testicular morphology 72h after a single oral dose of mDNB showing (a) vacuolation (arrowheads) of the epithelium of numerous seminiferous tubules in addition to the irregular appearance of round spermatids (x100); (b) section through three adjacent seminiferous tubules containing multinucleated giant bodies (arrowheads) and a severely vacuolated epithelium (arrowhead) (x190); (c) section through three adjacent seminiferous tubules, the lower two containing numerous degenerating germ cells whilst in the upper tubules the round spermatids appear grossly abnormal (arrows) (x190).

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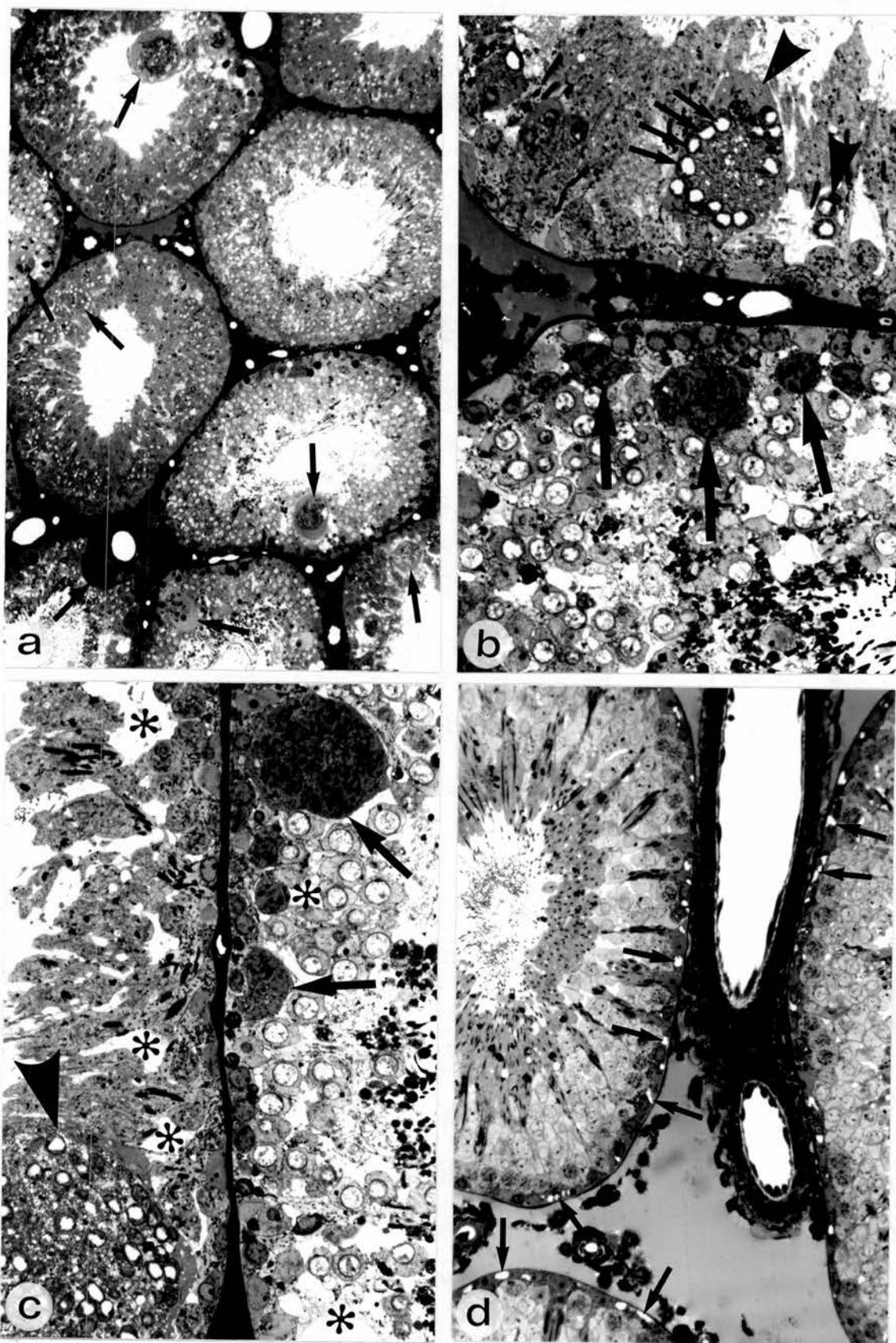
Plate 3.5. Testicular morphology 72h after a single oral dose of mDNB showing (a) multinucleated giant bodies (arrows) in the epithelium (x75); (b) section through two adjacent seminiferous tubules illustrating multinucleated giant bodies containing pachytene spermatocytes (large arrows) or round spermatids (small arrows) within other giant bodies (arrowheads) (x300); (c) section through two adjacent seminiferous tubules showing giant bodies containing pachytene spermatocytes (arrows) or round spermatids (arrowheads) in addition to a vacuolated epithelium (astericks) (x300); (d) section through three adjacent seminiferous tubules illustrating vacuolation towards the base of the Sertoli cell (arrows) (x190).

epithelium and only occasional degenerating pachytene spermatocytes (plate 3.5 d).

At 7 days post-treatment all tubules in some mDNB treated animals were affected to varying degrees (plate 3.6 a). In most tubules round spermatids were completely absent while only a few elongate spermatids were observed and the population of pachytene spermatocytes was severely depleted, whilst those present had matured from germ cells which, at the time of exposure, were unaffected by mDNB administration (plate 3.6 b). Within some tubules multinucleated giant cells were still apparent (plate 3.7 a), whilst in other tubules the organisation of the epithelium had completely broken down, with Sertoli cell cytoplasm prominent at the edge of the tubular lumen (plate 3.7b and c). Again by contrast, testicular histology in other mDNB-treated animals appeared normal except for the appearance of a number of small vacuoles within the epithelium of a number of tubules (plate 3.7 d).

By 14 days post-treatment regeneration of the epithelium was apparent in most tubules in mDNB-treated rats which appeared to have been severely affected by mDNB (plate 3.8 a). Numerous tubules in different stages of the spermatogenic cycle contained pachytene spermatocytes and round spermatids, the latter being derived from pachytene spermatocytes observed at 7 days post-treatment; however elongate spermatids were completely absent from all tubules (plate 3.8 b). In contrast, within some tubules spermatogenesis appeared to have completely broken down in comparison to the regeneration observed in neighbouring tubules (plate 3.9 a). These tubules contained Sertoli cells, spermatogonia and a few pachytene spermatocytes. In addition to the changes outlined above, the seminiferous tubules in severely affected animals appeared smaller in cross sectional area in comparison to control sections and/or an increase in the interstitial fluid space appeared to have taken place (plate 3.9 b). Detailed morphometry is, however, required in order to determine if these effects are real or due to histological artifact.

At 21 days after treatment with mDNB the seminiferous epithelium in most tubules appeared normal with the exception of missing elongate spermatids in many stages and vacuolation of the epithelium (plate 3.10 a). Within tubules at stage VIII residual body formation had occurred. In contrast to the tubules which contained a regenerating epithelium, others were either completely atrophic or were only just beginning the regenerative process (plate 3.10 b).

By 28 days post-treatment most tubules either appeared normal with an intact epithelium containing all germ cell complements or exhibited
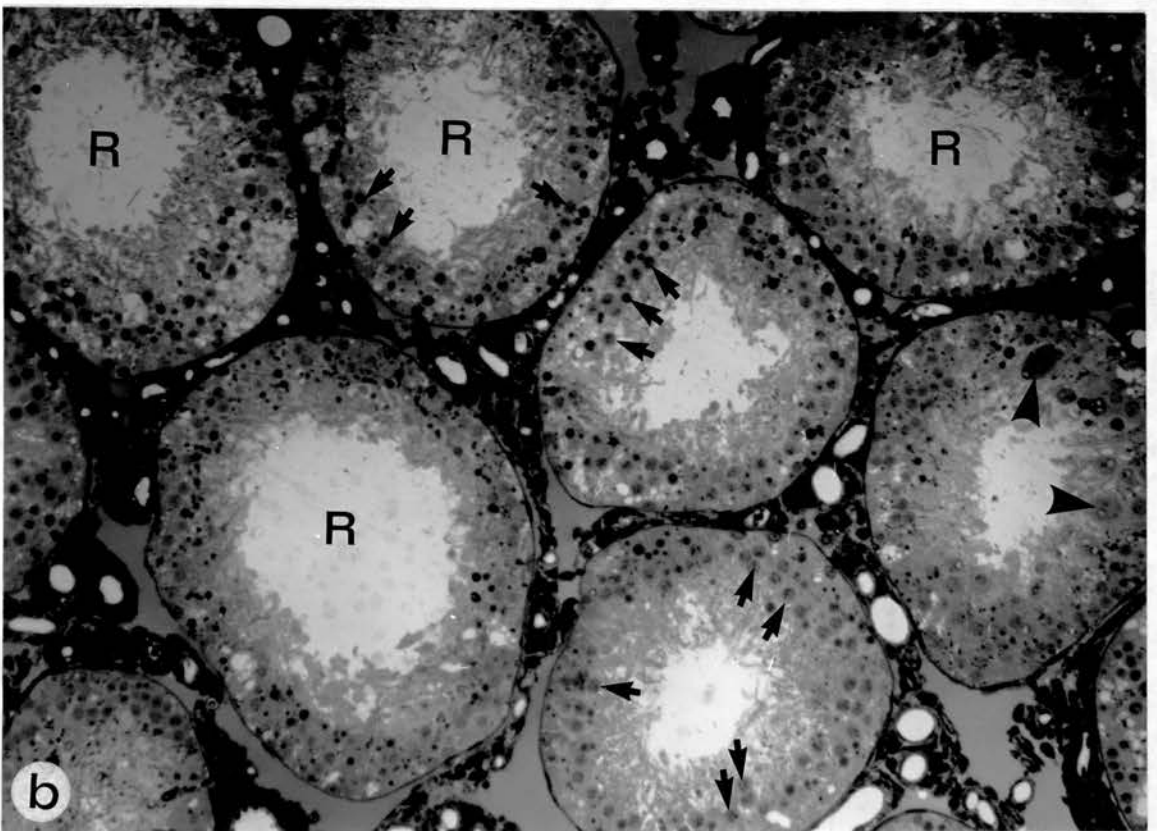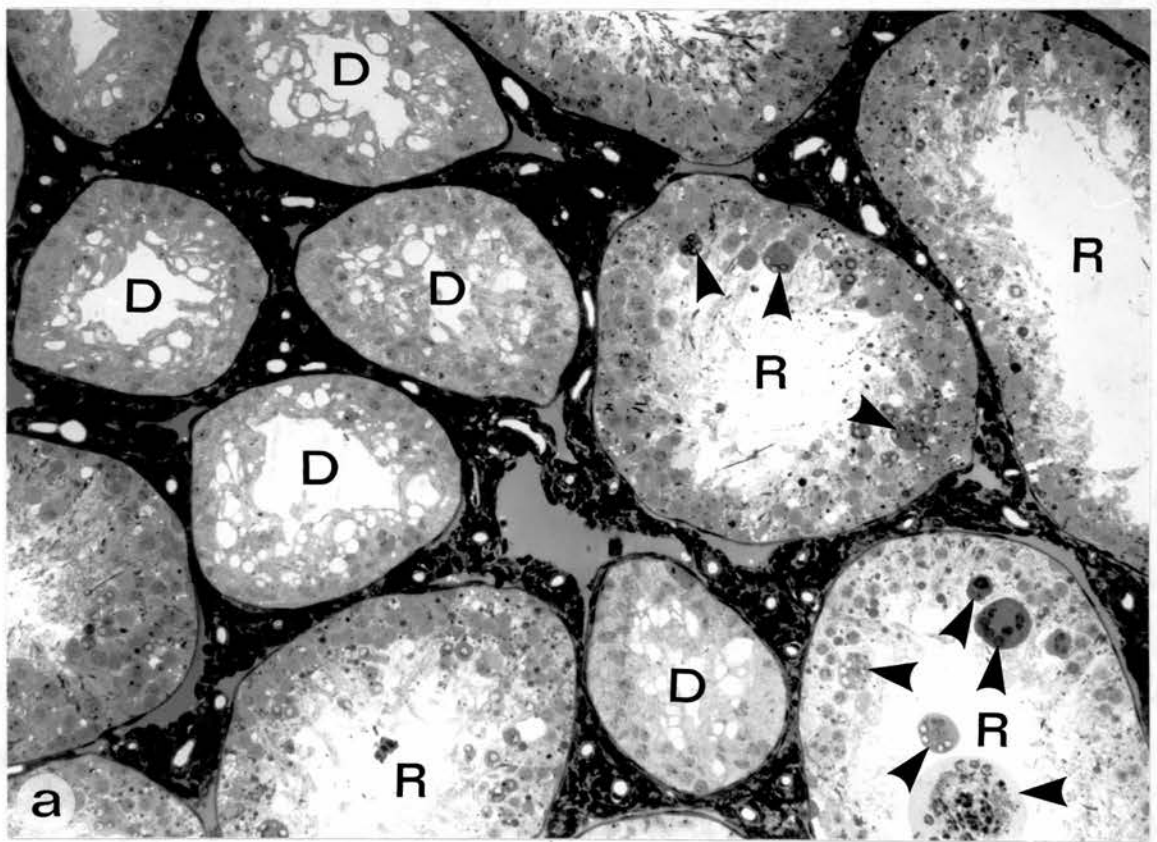
Plate 3.6. Testicular morphology 7 days after a single oral dose of mDNB showing (a) numerous tubules (D) containing only Sertoli cells or Sertoli cells plus a few spermatogonia whilst in the epithelium of other tubules, multinucleated giant bodies (arrowheads) (x100); (b) numerous tubules exhibiting regeneration of the seminiferous epithelium together with giant bodies (arrowheads); arrows show pachytene spermatocytes (x100). In (a) note also the apparent hyperplasia of the interstitial tissue.
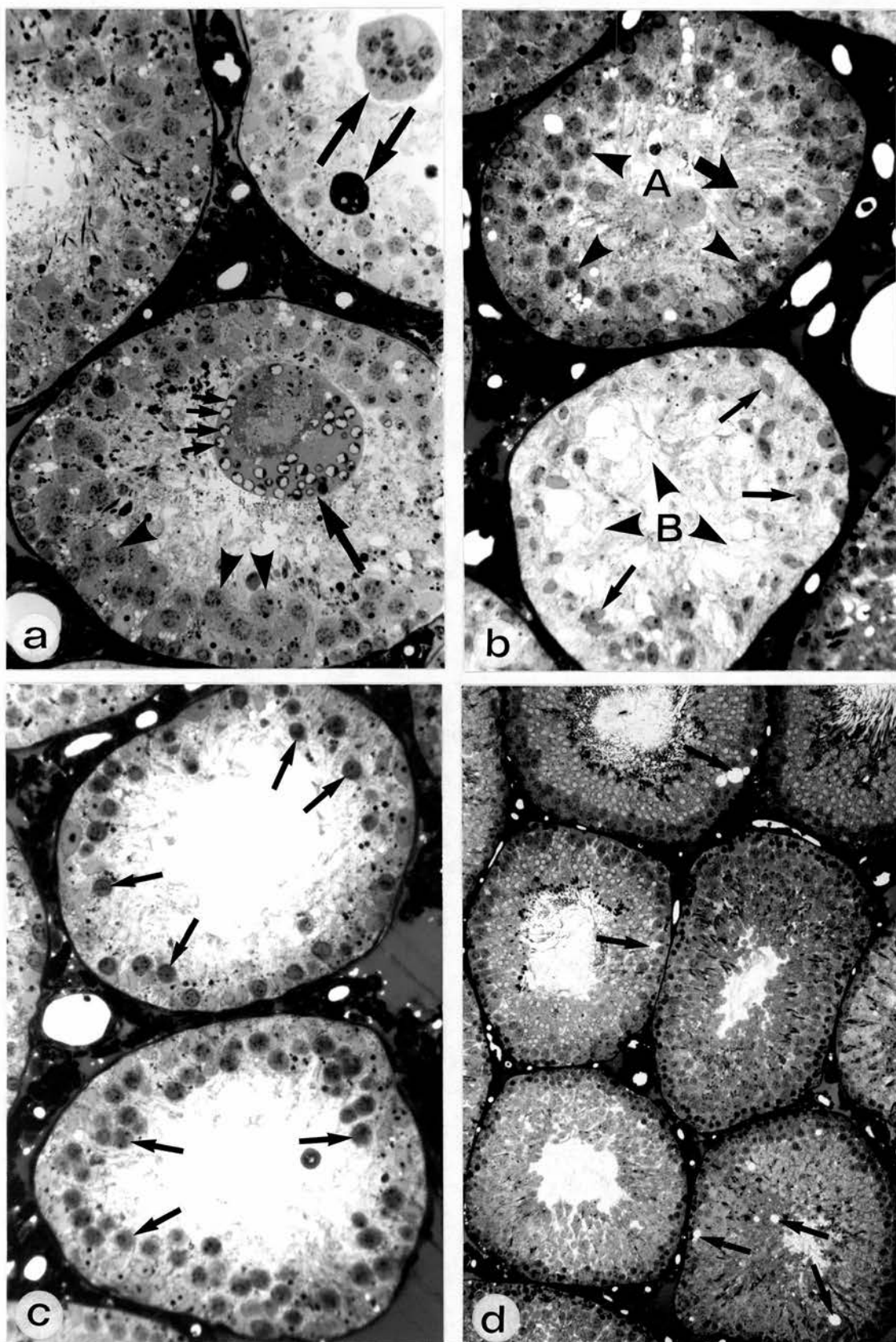
Plate 3.7. Testicular morphology 7 days after a single oral dose of mDNB showing (a) three adjacent seminiferous tubules showing multinucleated giant cells (large arrows) containing degenerating round spermatids (small arrows) in addition to normal pachytene spermatocytes within the same epithelium (arrowheads) (x190); (b) two adjacent seminiferous tubules, one of which has a regenerating epithelium (A) containing pachytene spermatocytes (arrowheads) whilst the other tubule (B) contains only Sertoli cells and occasional spermatogonia (arrows): Note the extensive cytoplasm of the Sertoli cells (arrowheads) (x190); (c) section through two adjacent seminiferous tubules which contain only pachytene spermatocytes (arrows) besides Sertoli cells and spermatogonia (x190) (d) low power view of seminiferous tubules relatively unaffected by mDNB 7 days post-treatment containing vacuoles within the epithelium (arrows): Note the appearance of the tubule (bottom right) half of which is at stages II-III and the other half at stage VI (x75).   98
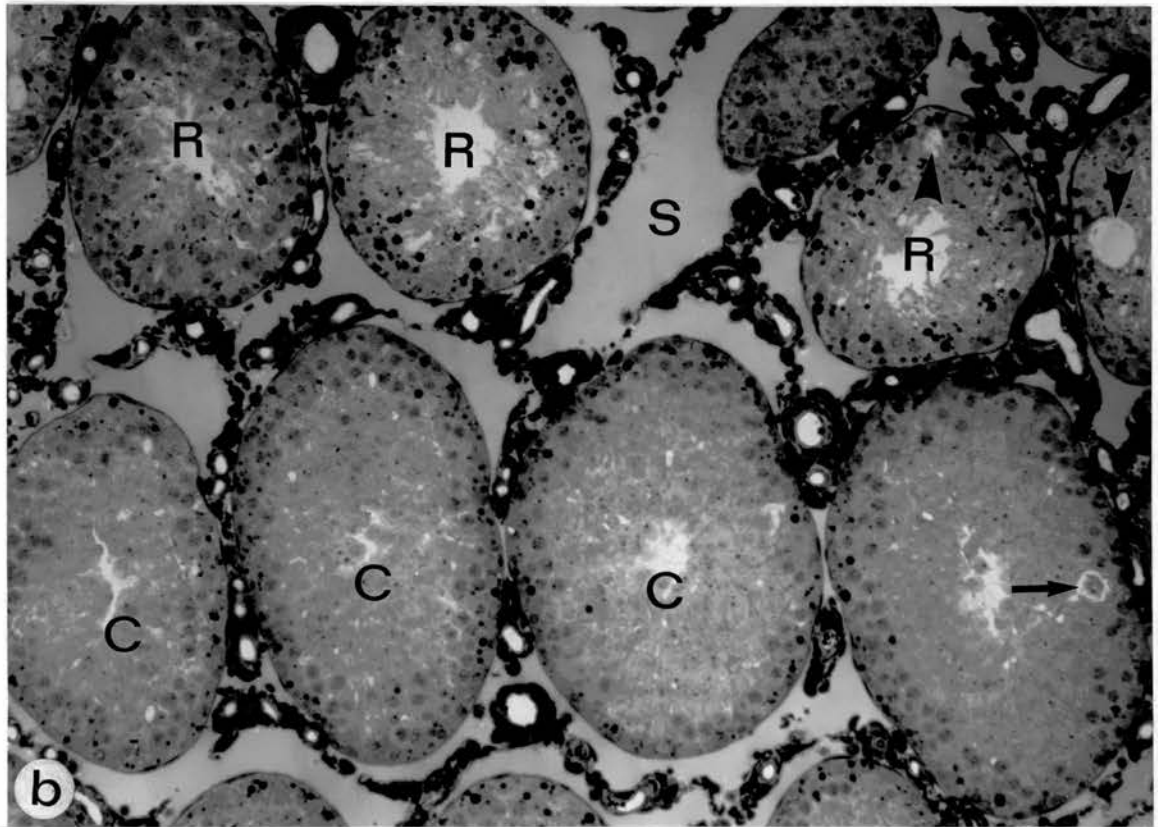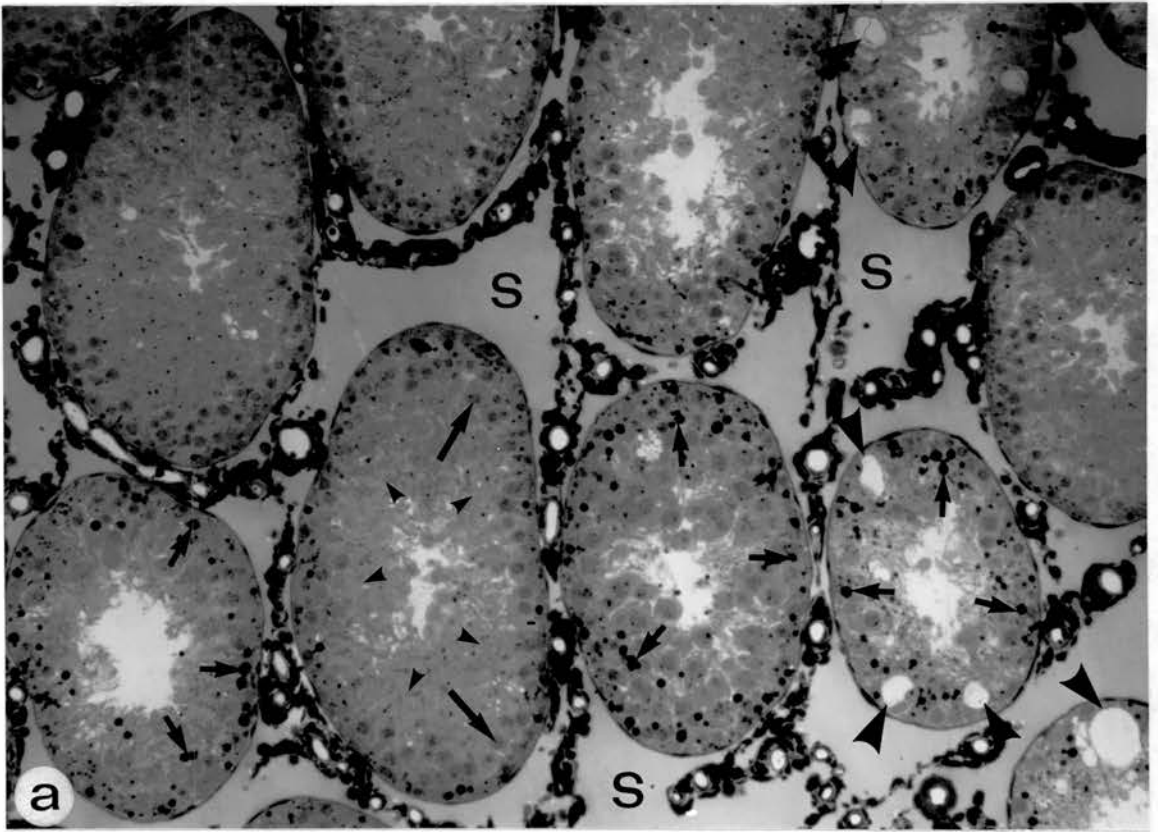
Plate 3.8. Testicular morphology 14 days after a single oral dose of mDNB showing (a) seminiferous tubules with a regenerating epithelium containing pachytene spermatocytes (large arrows) and round spermatids (small arrowheads), but no elongate spermatids; in some tubules the epithelium also contained degenerating germ cells (small arrow): arrowheads = vacuoles. Note also the abnormal expansion of the interstitial space (S) (x100); (b) comparison of seminiferous tubules exhibiting a regenerating epithelium (C) with other tubules (R) in which regeneration has hardly occurred: Note the abnormal expansion of the interstitial space(s). Arrowhead = vacuoles, arrow = degenerating germ cells (x100).

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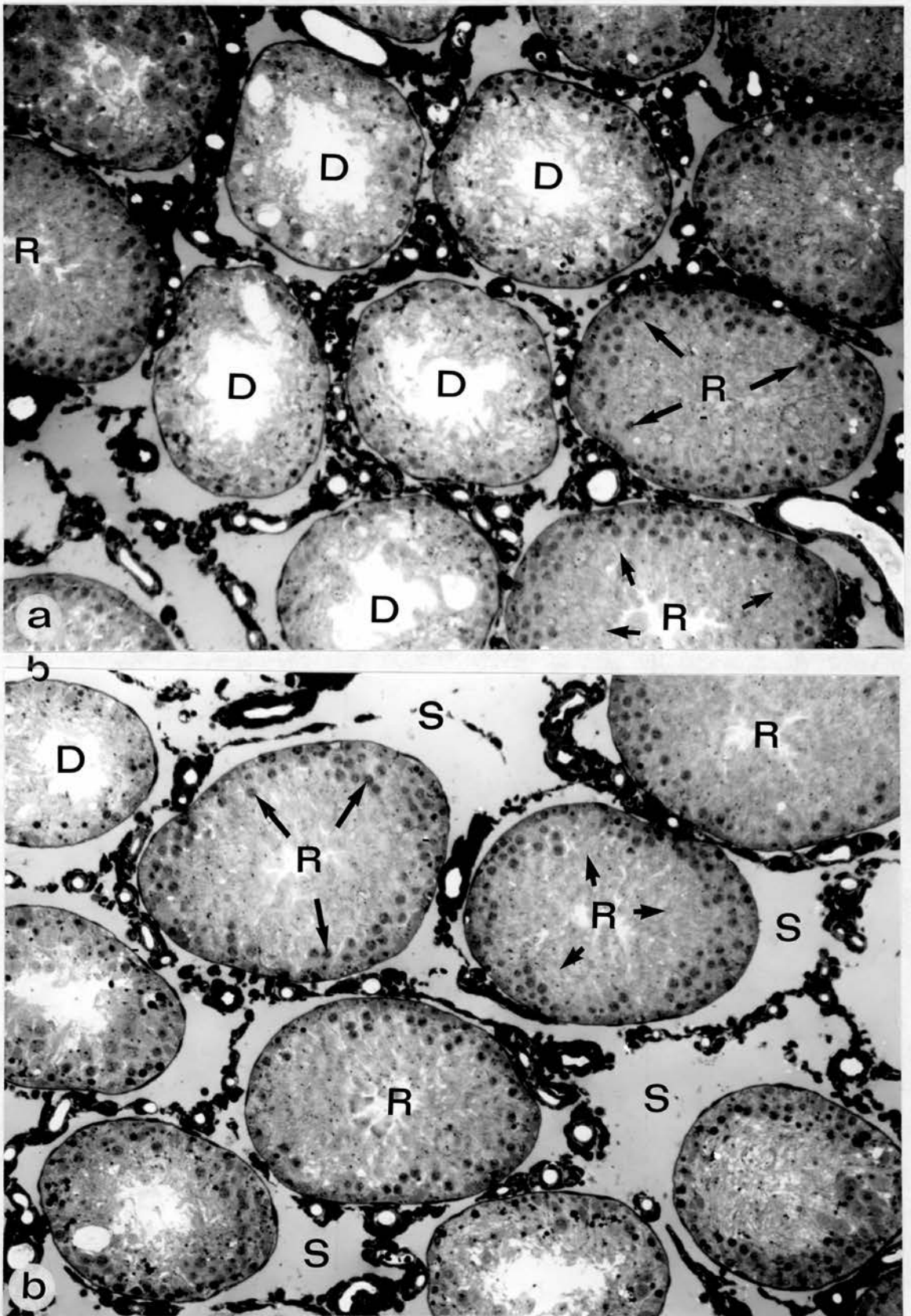
Plate 3.9. a & b. Testicular morphology 14 days after a single oral dose of mDNB showing (a) seminiferous tubules with a well-developed, regenerating epithelium (R) alongside atrophied tubules (D) containing only Sertoli cells and a few early germ cells: Note also the abnormally expanded interstitial space(s) large arrows = pachytene spermatocytes; small arrows = round spermatids (x100).

100
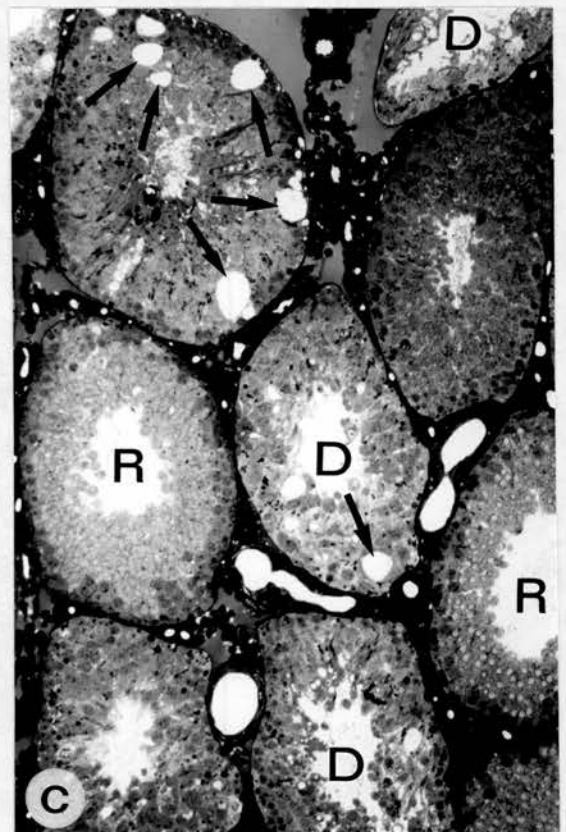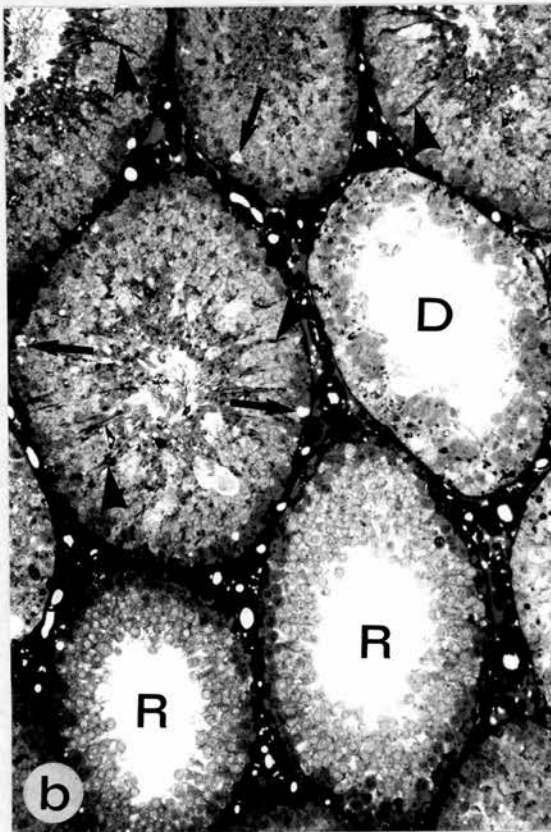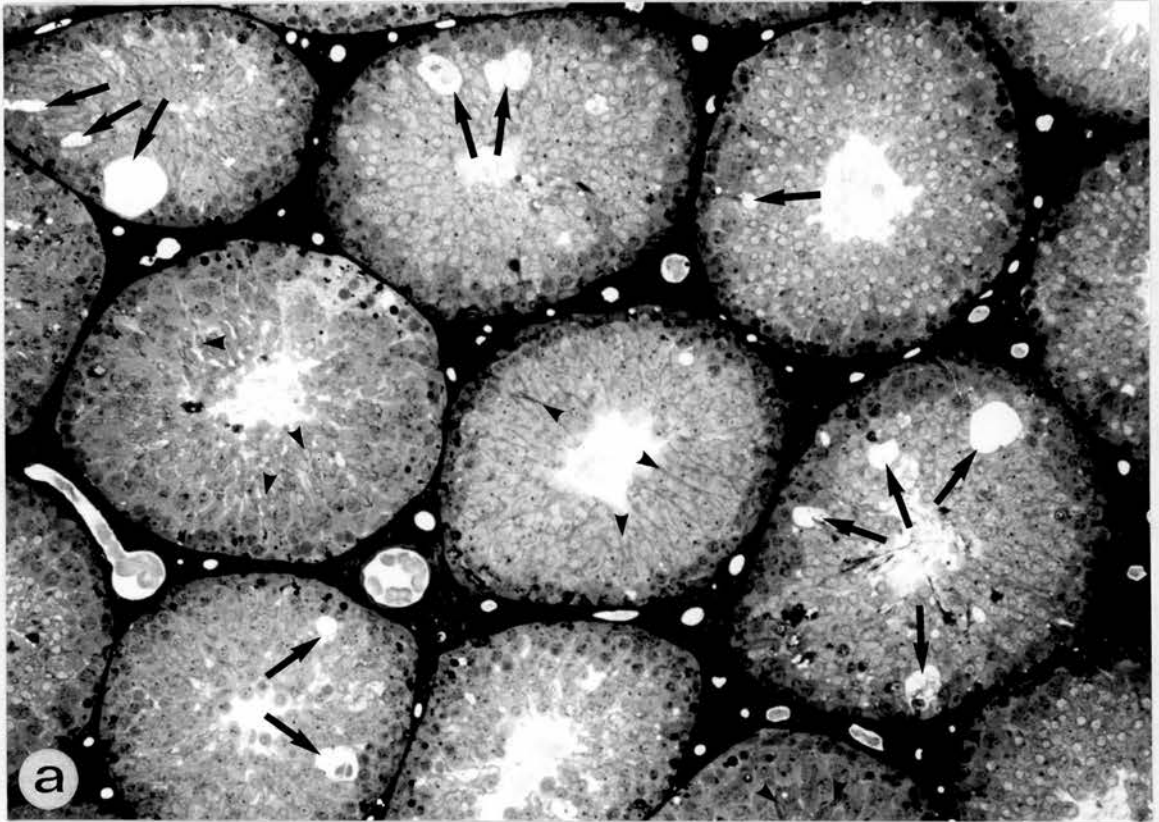
Plate 3.10. Testicular morphology 21 days after a single oral dose of mDNB showing (a) seminiferous tubules exhibiting marked regeneration of the epithelium but still with abnormal vacuolation (arrows): Note that the occasional elongate spermatid is now evident (arrowheads) (x100); (b and c) low power views exhibiting varying degrees of regeneration of the epithelium (R), alongside a severely atrophied tubule (D); note the presence of elongate spermatids (arrowheads) in some tubules; arrow = vacuole (x75).

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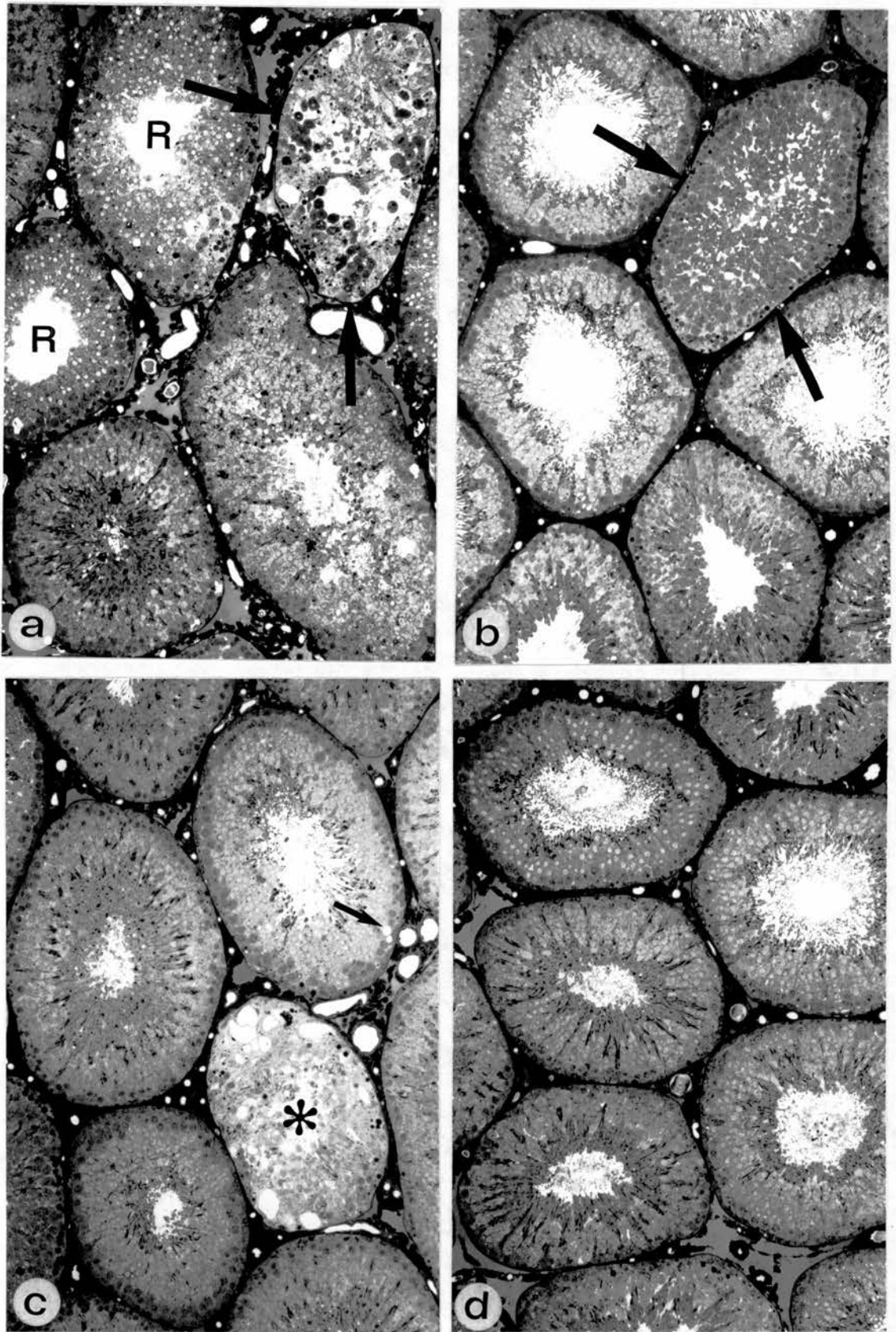
Plate 3.11. (a) Testicular morphology 28 days after a single oral dose of mDNB showing (a) seminiferous tubules in various stages of regeneration together with a severely atrophied tubules (arrows) containing only Sertoli cells and degenerating germ cells; (b) testicular morphology 42 days after a single oral dose of mDNB illustrating qualitatively normal spermatogenesis in most tubules but a disrupted epithelium in a neighbouring tubule (arrows); (c) testicular morphology 70 days after a single oral dose of mDNB showing qualitatively normal spermatogenesis in most tubules but severe atrophy in a neighbouring tubule (asterick); arrow shows vacuoles; (d) testicular morphology in a control animal 70 days after treatment illustrating normal spermatogenesis in all tubules (x75).

varying degrees of disruption ranging from complete atrophy to a vacuolated or disorganised epithelium (plates 3.10 c and 3.11 a) together with tubules which were in different phases of regeneration. By 42 days and at 70 days post-treatment most tubules contained a completely normal complement of germ cells (see plates 3.11 b and 3.11 c respectively). However within some tubules the epithelium still appeared disorganised (plate 3.11 b) while others were completely atrophic or contained only Sertoli cells, spermatogonia and some pachytene spermatocytes (plate 3.11 c) in comparison to controls (plate 3.11 d).

3.4.7. Effect of mDNB on Sertoli cell morphology *in vitro* and germ cell exfoliation.

Control cultures of Sertoli cells and Sertoli cells plus germ cells were considered to be normal under histological examination 24h after the administration of DMSO to the cultures (plate 3.12 a). Confluent monolayers of Sertoli cells were formed in culture within 3 days of plating. Co-cultures appeared morphologically normal with no apparent germ cell necrosis or significant observable exfoliation of germ cells or Sertoli cells induced by the vehicle (plate 3.12 a). Sertoli cell mono-cultures also appeared confluent following DMSO administration with no observable vacuolation within Sertoli cells (not shown).

Following exposure of Sertoli cell mono-cultures or Sertoli cell-germ cell co-cultures to mDNB for 24h, dose-dependent morphological changes were observed in culture (plate 3.12 b, c and d). In mono-cultures and co-cultures Sertoli cell vacuolation was observed (plate 3.12 c and d). In the latter it coincided with an observable decrease in the density or absence of different populations of germ cells (plate 3.12 c and d). These effects were evident from visual examination of the mono and co-cultures exposed to mDNB at doses up to 10^{-3} M (plate 3.12 d), when compared visually with vehicle-treated control cultures (plate 3.12 a). Sertoli cell vacuolation in mono- and co-cultures appeared dose-dependent, occurring to a greater extent at doses in excess of 5×10^{-4} M (plate 3.12 c). Morphological examination of co-cultures exposed to doses of mDNB in excess of 5×10^{-4} M exhibited Sertoli cell phagocytosis of a number of degenerating germ cells of different classes (plate 3.12 c and d). In both mono-culture and co-culture the administration of 5×10^{-3} M mDNB resulted in gross morphological changes (not shown). These included a breakdown of the confluent monolayer of Sertoli cells resulting in the appearance of large holes in the culture together with significant vacuolation
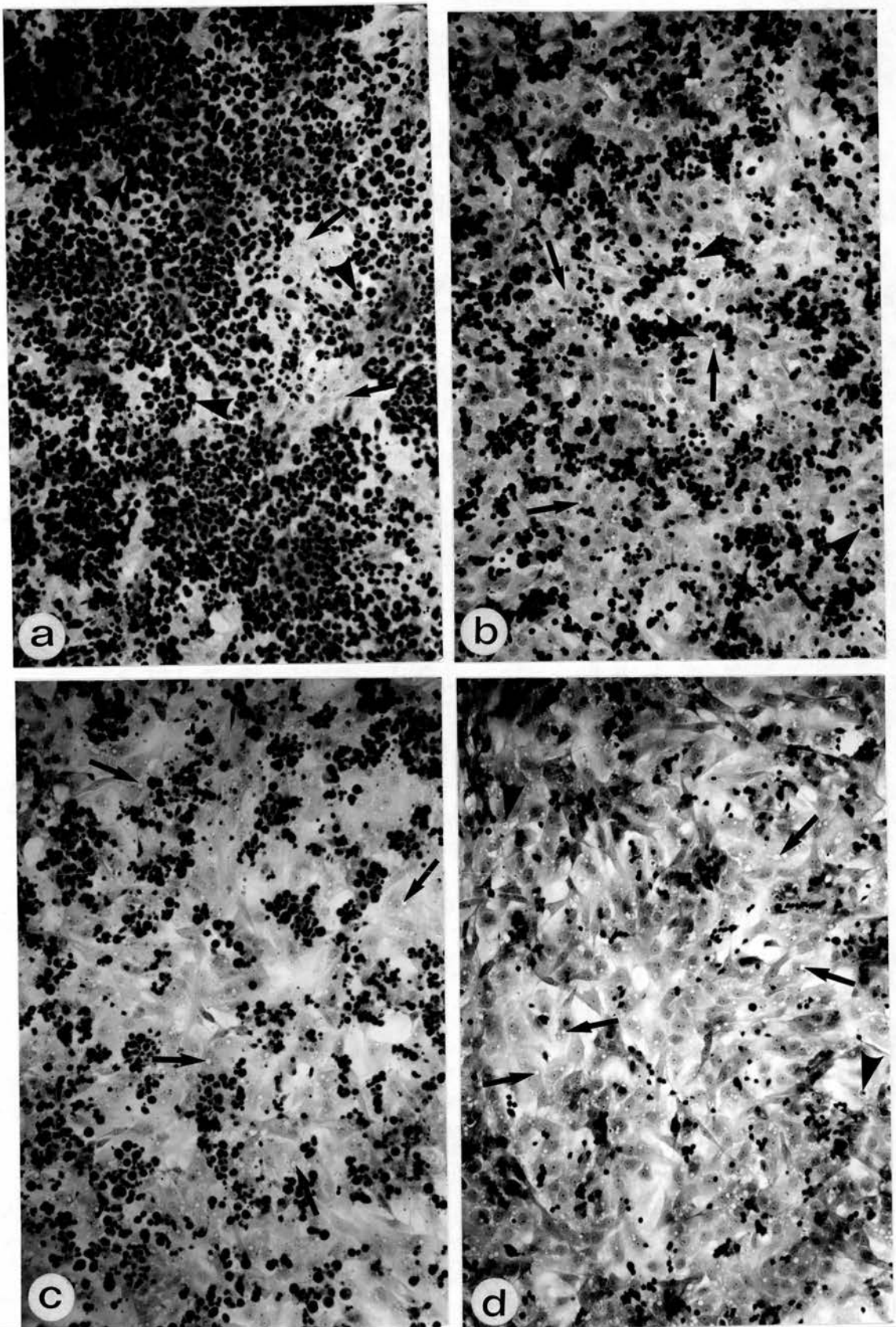
Plate 3.12. Sertoli-germ cell co-cultures following exposure for 24h to; (a) control vehicle (DMSO-0.3% v/v), showing confluent Sertoli cells (arrows) beneath numerous adherent clusters of germ cells (arrowheads); (b) 5×10^{-6}M mDNB, showing a noticeable decrease in the density of adherent germ cells; (c) 5×10^{-4}M mDNB; illustrating phagocytosis of degenerating germ cells by Sertoli cells (arrowheads), vacuolation within Sertoli cells in the confluent monolayer (arrows) and a marked decrease in the density of adherent germ cells; (d) 10^{-3}M mDNB, showing significant vacuolation of Sertoli cells (arrows) and phagocytosis of degenerating germ cells (arrowheads); note that the vast majority of adherent germ cells have been lost. Haematoxylin and Papanicolaou's (x75).

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within both Sertoli cells and germ cells of different types in addition to the absence of most germ cells in co-culture.

The effect of mDNB administration on germ cell exfoliation from Sertoli cell-germ cell co-cultures is shown in Fig 3.5. Following exposure to mDNB for 24h, a dose-dependent exfoliation of cells was observed (Fig 3.5). The exfoliation of germ cells induced by mDNB was significantly greater (P<0.001) than that measured in control DMSO-treated co-cultures at doses in excess of 0.5 µM $(5\times10^{-7}M)$ mDNB. These exfoliated cells were considered to be primarily germ cells since treatment of co-cultures with $1\times10^{-4}M$ (100µM) mDNB resulted in a significant exfoliation of cells ($42.5\times10^{-4} \pm 4.6\times10^{4}$ total cells/well - this study and see Chapter 4, Fig 4.7) whereas an insignificant number of exfoliated cells ($2.3\times10^{-3} \pm 0.7\times10^{-3}$ total cells/well) were measured after exposure of Sertoli cell mono-cultures to an identical dose of mDNB. In the latter instance, the exfoliated cells represented only 0.6% of the cells on the plate and are presumed to represent the detachment of residual germ cells that contaminate Sertoli cell cultures following Tris treatment. Control co-cultures treated with vehicle (DMSO) showed a significant (P<0.01) increase in detachment of cells when compared to non-treated co-cultures.

In DMSO-treated cultures, $95 \pm 1\%$ (mean ± SD) of the exfoliated cells excluded trypan blue, indicating that they were essentially viable. After exposure to low doses of mDNB (up to $1\times10^{-6}M$ or 1.0 µM on Fig 3.5), the viability of exfoliated cells was similarly high (90-93%), but it decreased marginally with higher doses of mDNB [$88 \pm 3\%$ at $1\times10^{-5}M$ (10 µM) and $81 \pm 4\%$ at $1\times10^{-4}M$ (100 µM)] and was decreased significantly at $1\times10^{-3}M$ (1000µM) ($73 \pm 3\%$) and substantially ($56 \pm 7\%$) after exposure of co-cultures to $5\times10^{-3}M$ (5000 µM) mDNB (see Table 3.1. for details); the latter dose was thus considered as cytolethal for germ cells.
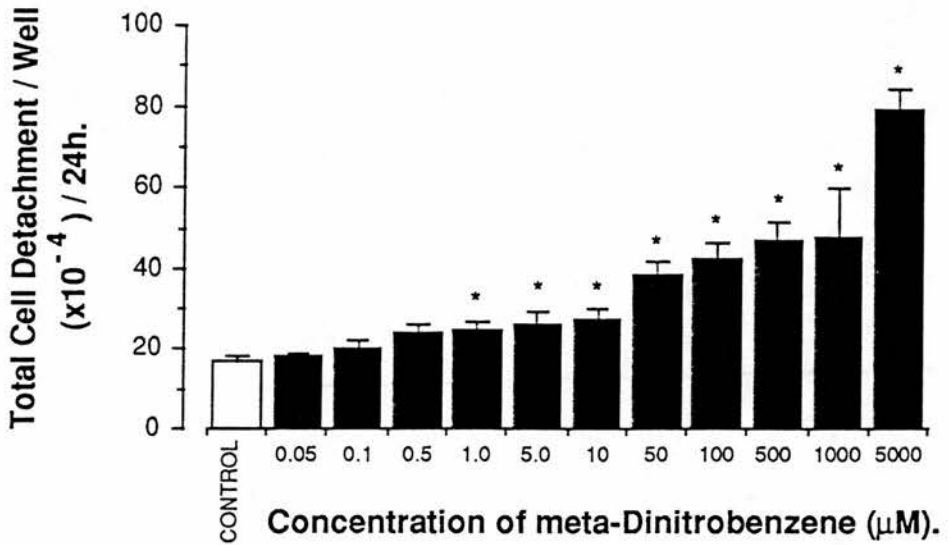
Fig 3.5. Effect of a range of doses of *meta* dinitrobenzene (■) or vehicle alone (DMSO - 0.3% v/v) (☐) on the number of detached cells present in the medium of Sertoli cell-germ cell co-cultures following a 24h exposure period. Values are means ± SD for 6 replicates (*P<0.001, in comparison to control). Results of a representative experiment are shown.

Table 3.1. The effect of a range of doses of mDNB (0.05 - 5000 µM) or control vehicle (DMSO - 0.3% v/v) on the viability of exfoliated cells removed from the medium of co-cultures 24h after compound administration. Cells were counted using a haemocytometer and non-viable cells were identified using 0.05% v/v trypan blue solution; cells which excluded trypan blue were considered viable. Values are means ± SD for six replicate cultures (*P<0.01,**P<0.001, compared to control). Results of a representative experiment are shown.

Compound and dose (µM)	Percentage of exfoliated cells absorbing trypan blue dye (mean ±SD).
DMSO (0.3%v/v)	4.8 ± 1.1
mDNB 0.05	3.7 ± 0.9
mDNB 0.1	6.3 ± 1.8
mDNB 0.5	5.2 ± 2.2
mDNB 1.0	6.9 ± 2.7*
mDNB 5.0	9.1 ± 3.0*
mDNB 10	11.5 ± 3.5*
mDNB 50	15.7 ± 4.2**
mDNB 100	18.8 ± 3.9**
mDNB 500	23.8 ± 4.8**
mDNB 1000	26.2 ± 3.4**
mDNB 5000	43.6 ± 6.9**

3.4.8. Effect of mDNB on inhibin secretion by Sertoli cells in culture.

The exposure of both mono-cultures and co-cultures to mDNB resulted in a biphasic response of basal inhibin secretion (Fig 3.6). In Sertoli cell cultures an intermediate range of doses of mDNB (1×10^{-6} to 5×10^{-4}M) stimulated inhibin secretion to a significant extent in comparison to control; the highest levels in this range of doses were measured after the administration of 1×10^{-5}M mDNB. In co-culture, inhibin secretion was also stimulated at 1×10^{-5} and 5×10^{-5}M mDNB in comparison to the respective control (Fig 3.6). In both mono- and co-cultures 1×10^{-4}M mDNB had no effect on Sertoli cell inhibin secretion. An increase in inhibin secretion was also observed following the administration of high doses (1×10^{-4} and 5×10^{-4}M) of mDNB to both mono- and co-cultures, while that induced by the latter concentration of mDNB was the largest increase observed in respective mono- and co-cultures. In addition 5×10^{-3}M mDNB induced a significant decrease in basal inhibin secretion by Sertoli cell mono-cultures (Fig 3.6).
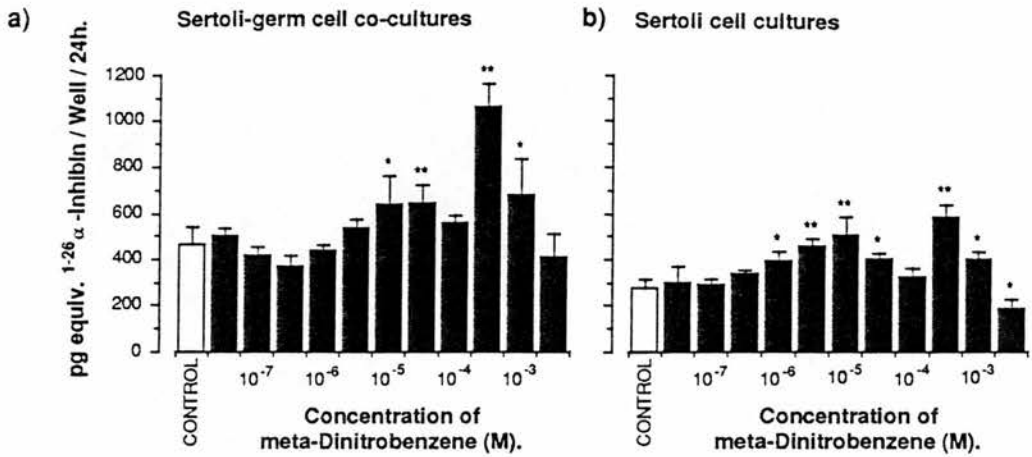
Fig 3.6. Effect of a range of doses of *meta* dinitrobenzene (■) or vehicle alone (DMSO - 0.3% v/v) (☐) on the secretion of $^{1-26}\alpha$-inhibin by Sertoli cell-germ cell co-cultures (**a**) or Sertoli cell cultures (**b**) over a 24h exposure period. Values are means ± SD for 6 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a representative experiment are shown.
Note however that the Sertoli cell cultures and co-cultures are derived from separate cell preparations. Labels for intermediate doses of *meta* dinitrobenzene on the abscissa (eg 5×10^{-8} to 5×10^{-3} M) have been omitted for clarity.

This biphasic response of Sertoli cells to mDNB was similar to that observed following NB administration (Chapter 4) and was observed in 2 out of 3 separate culture preparations (data not shown). Indeed the consistent response in basal inhibin secretion obtained following the administration of mDNB to mono- and co-cultures at a concentration of 10^{-4} M was observed not only in these experiments but also in other *in vitro* experiments (see Chapter 4) utilising this dose but using separate culture preparations. Although the biphasic pattern of inhibin secretion induced by mDNB was consistent and, in general, independent of the absence or presence of germ cells, the magnitude of inhibin secretion in both toxicant-treated (at most doses) and control cultures was consistently and significantly ($P<0.01$) greater in Sertoli cell-germ cell co-cultures than in cultures of Sertoli cells alone (Fig 3.6).

3.5. DISCUSSION.

The results of the present study confirm those published to date which suggest that mDNB is a testicular toxicant, and add further evidence to the possibility that mDNB induces testicular toxicity through a direct action on the Sertoli cell. In addition, studies presented here are amongst the first to correlate temporal changes in hormonal alterations to testicular morphological events which follow mDNB administration. Furthermore, the effects of mDNB on the Sertoli cell have been investigated *in vitro* using known indices of Sertoli cell function including germ cell exfoliation, culture morphology and, in addition, a new and potentially useful index of Sertoli cell function *in vitro* and *in vivo*, that of inhibin secretion.

Over the past decade or so numerous investigations have taken place into the reproductive toxicity of mDNB (Cody *et al*, 1981; Blackburn *et al*, 1985; 1988; Foster *et al*, 1986; 1989; Linder *et al*, 1986; Rehnberg *et al*, 1988; Hess *et al*, 1988). All of these data suggest that oral administration of mDNB to adult male rats in either acute or chronic dose regimes produces seminiferous tubule dysfunction, altered serum and testicular hormone levels, leading ultimately to a reduction in or loss of fertility which is partially or wholly reversible. Further experiments performed *in vitro* using Sertoli cells cultured in the presence or absence of germ cells have confirmed that the Sertoli cell is the initial target of mDNB and it is this effect that leads to the subsequent testicular disruption (Foster *et al*, 1986, 1987; Lloyd & Foster 1987; Williams & Foster 1988).

In this study, the effects of mDNB on the rat testis were identified at the histological level within 24h of treatment. These initial effects were

manifested as Sertoli cell vacuolation together with the loss or degeneration of pachytene spermatocytes in seminiferous tubules at stages VII to XII, leading to a severely disorganised epithelium. The mechanism for the initial stage-specificity of this action is unknown, however other compounds have been shown to have rapid degenerative effects on pachytene spermatocytes. These include ethylene glycol mono-methyl ether (EGME) (Chapin *et al*, 1984; Creasy et al, 1985; 1986) nitrobenzene (Bond *et al*, 1981 and see Chapter 4) and an imidazole compound (De Martino *et al*, 1975). At 72h after mDNB treatment histological observations revealed that, in addition to the loss or degeneration of pachytene spermatocytes at stages VII to XII, the remaining population of these cells at stages I to VI were in the process of degeneration. In association with pachytene spermatocyte degeneration many round spermatids at stages I to VIII of the spermatogenic cycle appeared abnormal in comparison to controls. A number of these round spermatids appeared pyknotic and were in the process of degeneration while others had an opaque cytoplasm and fragmented nucleus. Multinucleated giant cells, the product of the coalescence of degenerating pachytene spermatocytes or round spermatids were also observed 72h post-treatment. These giant bodies were composed of either pachytene spermatocytes or round spermatids but in no section were pachytene spermatocytes and round spermatids observed within the same giant body. Giant body formation is a common occurrence during testicular dysfunction induced by several toxicants, and has previously been reported to occur after mDNB administration (Blackburn *et al*, 1985, 1988; Hess *et al*, 1988). Furthermore, multinucleated giant cells have been observed in the lumen of the caput epididymis following mDNB administration (Hess et al, 1985). These initial effects, including giant body formation were consistent with the previously reported findings identified at a histological level in the rat by Blackburn et al, (1985; 1988) and Hess *et al*, (1988), over the first 72h post- mDNB treatment.

In parallel with these testicular changes over the first 72h post-treatment, the hormonal milieu in identically treated rats was altered significantly from vehicle treated controls at 24 and 72h post-treatment. In particular, the levels of inhibin in testicular IF were raised significantly at 1 and 3 days post-treatment. Historically, inhibin has been postulated to negatively regulate pituitary FSH secretion whilst FSH positively regulates inhibin secretion. Applying this hypothesis, serum levels of FSH should have decreased significantly within 24h of treatment consistent with other findings (see nitrobenzene-Chapter 4) and in parallel with the substantial loss of germ cells and associated testicular dysfunction (de Krester & Kerr, 1983). However,

serum FSH levels were increased significantly at 3, 7 and 14 days post-treatment. Increases in serum FSH levels following mDNB administration have also been observed by Rehnberg *et al,* (1988) but only at 14 days post-treatment. Recent evidence suggests that in the adult male rat inhibin levels usually increase and decrease in parallel with changes in FSH (Lincoln, 1989) and this certainly seems to be the case following NB administration (see Chapter 4). However, following mDNB administration IF inhibin levels were increased at 1 and 3 days post-treatment while serum FSH levels were not increased until 3 and 7 days post-treatment. This is in contrast to findings in other experimental situations (Sharpe *et al,* 1988; Sharpe & Maddocks, 1988; 1990). Alternatively, it is possible that inhibin secretion into IF may have been increased as a direct result of the action of mDNB on the Sertoli cell, although the present *in vitro* studies do not provide clear cut evidence for this effect. A further possible explanation is that the bidirectional secretion of inhibin may have changed such that more is secreted into testicular IF. Recent evidence suggests that the levels of inhibin in testicular IF often, but not always, change in parallel with alterations in peripheral blood levels (Maddocks & Sharpe, 1990). However the main route by which inhibin reaches the peripheral circulation in the adult rat is not via testicular IF but by secretion into seminiferous tubule fluid with resorption into blood from the rete testis via the mediastinal venous plexus overlying the rete (Maddocks & Sharpe, 1989; 1990). Changes in the route of secretion of inhibin occur in the rat during sexual maturation and probably after experimental disruption of spermatogenesis, therefore IF levels of inhibin may not always parallel changes identified in peripheral blood (Maddocks & Sharpe, 1989; 1990). Since the Sertoli cell is known to secrete many products in a bidirectional manner (Sharpe, 1988) including inhibin (Baker *et al*, 1978) it is possible that mDNB may alter the route of secretion of inhibin such that proportionately more is secreted into IF. Unfortunately at the time of the experiment, samples of serum and not plasma were collected from mDNB-treated rats. Recent studies have demonstrated that products of haemolysis in serum cross react in the inhibin RIA which make it impossible to measure inhibin levels in serum.

In addition to fluctuations in the levels of inhibin in testicular IF and serum FSH 72h after treatment the levels of serum LH and serum and IF testosterone were also altered, in some cases significantly, over this period post-treatment. In particular the levels of serum and IF testosterone were significantly elevated, the former at 24h post-treatment and the latter at 24 and 72h post-treatment. However, serum LH levels increased in a time-dependent

manner over the first 14 days post-treatment, increasing in a pattern similar to that observed for serum FSH and reaching a zenith at 14 days post-treatment. The significant increases in serum and IF testosterone levels evident at 24 and 72h post mDNB treatment were not observed by Rehnberg *et al* (1988) in a similar experiment in which the endocrine status of male rats killed at 24h and at 7 and 14 days was evaluated following a single oral dose of 32mg/kg of mDNB. They observed significant increases in testosterone levels in IF and seminiferous tubule fluid at 7 and 14 days post-treatment and a transient decrease in serum testosterone levels 24h after treatment, however no significant alterations in serum LH levels were evident up to 14 days post-treatment. The discrepency between the published results of Rehnberg *et al* (1988) and those quoted here my be explained by the marked, between-animal fluctuations in testosterone levels observed in these studies. Such fluctuations may account for the significant rise in IF and serum testosterone levels observed at 24 and 72h post-treatment. Alternatively, since LH levels are unaffected by mDNB administration, increases in testosterone levels may be a direct result of the effects of mDNB on androgen production by Leydig cells. Since no-one has demonstrated any direct effects of mDNB on Leydig cells to date, this seems unlikely. However there is a possibility that, as a result of Sertoli cell dysfunction, Sertoli cell-Leydig cell paracrine communication may be disrupted leading to an enhancement of Leydig cell androgen biosynthesis through mechanisms independent of the level of serum LH or FSH. A number of investigators have demonstrated not only the importance of androgens for normal spermatogenesis to occur but the importance of possible paracrine control of Leydig cell androgen synthesis by Sertoli cells *in vivo* (reviewed in Sharpe, 1986). Further work is required to evaluate such a possibility.

At 7 days post-treatment, histological observations of testes taken from mDNB-treated animals showed that a significant compression of the epithelium had occurred. These seminiferous tubules were completely denuded of round and many elongate spermatids, the former as a direct consequence of mDNB perturbing Sertoli cell function while the latter being a result of both maturation depletion of the epithelium and probably the premature release and/or phagocytosis of elongate spermatids. In animals severely affected by mDNB, spermatogenesis had completely broken down in all seminiferous tubules, and the epithelium appeared very disorganised. These effects of mDNB identified at the histological level were consistent with previously reported findings (Blackburn *et al,* 1985; 1988; Hess *et al,* 1988) observed at 7 days post-treatment. At 14 days, regeneration, proliferation and re-organisation of the

epithelium had occurred. Germ cells which were unaffected by mDNB administration (ie spermatogonia, leptotene and zygotene spermatocytes), had matured to a point such that most seminiferous tubules contained pachytene spermatocytes and round spermatids, although elongate spermatids were still completely absent. In addition to these intratubular changes either the volume of the interstitial fluid compartment had visibly increased or the surface area of the seminiferous tubules had decreased. Since the volume of drip-collected IF was increased significantly at this time point it seems likely that the volume of IF had increased at this time interval post-treatment. Why the volume of IF should be altered at this time point remains unknown, although it could be the result of raised serum LH levels which have been demonstrated to significantly increase IF volume (Sharpe, 1983). Volume changes in testicular IF have also been observed following the administration of other testicular toxicants including MAA (Bartlett *et al,* 1988) and other treatments known to perturb testicular function e.g. local testicular heating (Bartlett & Sharpe, 1987).

In parallel with the significant increase in serum levels of FSH (and in LH) observed at 7 and 14 days post-treatment, and as a direct consequence of germ cell degeneration at earlier time intervals, testicular weight declined in a time-dependent manner reaching a nadir by 14 days post-treatment. The coincidence of raised serum FSH levels and major disruption of spermatogenesis is a well recognised feature (de Krester & Kerr, 1983).

At 21 days after exposure to mDNB, some tubules had a full complement of germ cells whilst others contained all germ cell types except elongate spermatids. This division was purely due to the stage of the spermatogenic cycle since tubules in certain stages contained elongating spermatids while others, because of maturation depletion induced by mDNB, only contained germ cell types up to round spermatids which had not yet initiated the process of elongation. Although in numerous tubules an epithelium containing most, if not all, germ cell types was observed, many tubules contained extracellular vacuoles probably as a result of germ cell degeneration although this was not observed extensively at this time point. While numerous tubules appeared histologically normal in comparison to controls, a small percentage of tubules appeared abnormal. Within these tubules, spermatogenesis appeared to have completely broken down: these tubules contained Sertoli cells, spermatogonia and occasionally pachytene spermatocytes although their numbers were reduced in comparison to controls and some appeared pyknotic. The degeneration or lack of re-initiation of spermatogenesis in a percentage of tubules following mDNB administration was also observed by Hess *et al*

(1988). In their study between 25% and in some animals up to 100% of tubules were atrophic at 21 days post-treatment. In this study and in that by Hess and co-workers these atrophic tubules were observed at all subsequent time points post-treatment and here up to 70 days post-treatment while in the study of Hess *et al* (1988) they were observed even at 175 days post-treatment. Hess and co-workers have postulated that these so-called 'Sertoli cell only' tubules are formed when, for as yet unknown reasons, certain tubules fail to reinitiate spermatogenesis, (possibly because normal cellular associations are not re-established within a certain amount of time) or the re-synchrony of this re-initiation becomes perturbed such that the process of spermatogenesis continues to break down within these tubules. Although this theory cannot be confirmed or countered by the present results a possible alternative is that Sertoli cells are more susceptable to the effects of mDNB at a particular stage of the spermatogenic cycle. These perturbed Sertoli cells do not recover completely and, as a result, the process of re-initiation and re-synchronisation of spermatogenesis which occurs in other tubules between 14 and 21 days post-treatment does not occur. Therefore, since the role of the Sertoli cell is to support and maintain germ cells, any deviation from or lack of these functions may result in tubules which appear abnormal a long time after toxicant administration.

In most tubules at 28, 42 and 70 days post-treatment all types of germ cells were present. However, in a small percentage of tubules spermatogenesis had completely broken down. The percentage of these tubules varied between animals and at different time intervals post-treatment similar to that reported by Hess *et al* (1988). These temporal changes in testicular morphology can be equated to changes in fertility which have been demonstrated to be significantly reduced following mDNB treatment (Foster, 1989). A time-dependent decrease was observed in the percentage pregnancy rate in mating trials of virgin female rats to male rats administered 5 daily doses of mDNB at 5 or 10 mg/kg/dose. The levels of percent females pregnant reached a nadir during the 21 to 35 day period post-treatment in the groups exposed to 10 mg/kg whilst in the 5 mg/kg group males were significantly sub-fertile at 28 and 42 days post-treatment. Animals regained normal fertility at 49 days in the lower dose group and at 70 days in the higher dose group in comparison to controls (Foster, 1989). These time-dependent alterations in fertility can be equated to the morphological changes observed between 1 and 70 days post-treatment. Between 1 and 21 days post-treatment the decreasing numbers of sperm remaining in the epididymis are represented by the decreased fertility index.

Morphological observations suggest that between 28 and 35 days post-treatment spermiation takes place in a number of regenerating epithelia resulting in sperm in the caput epididymis over this period. However, given an epididymal transit time of between 7 and 14 days sperm would reach the cauda and be evident in the ejaculate between 42 and 49 days post-exposure. The number of sperm would be dependent on the degree of degeneration induced by mDNB. By 70 days spermatogenesis appears qualitatively normal in most tubules and similarly the percentage of females pregnant was within the normal range (Foster, 1989).

Consistent with the re-initiation of spermatogenesis and re-generation of germ cells beyond 14 days post-treatment, testicular weight at 21 days post-treatment had increased but was still significantly below control levels and remained so, increasing time-dependently up to 70 days post-treatment. At 28 days after treatment and up to 70 days post-treatment serum FSH levels in mDNB-treated rats remained significantly higher than in controls. A possible reason for this could be due to the continued degeneration and atrophy of a percentage of tubules following mDNB-treatment while re-initiation and normal spermatogenesis continued in neighbouring tubules (discussed later). Although this is plausible since raised serum FSH levels are consistent with major germ cell degeneration (de Krester & Kerr, 1983), it seems unlikely since the proportion of atrophic tubules in comparison to normal or regenerating tubules is small.

In addition to raised serum FSH levels, significantly elevated levels of inhibin in testicular IF were evident at 28 days post-treatment while serum and IF testosterone levels were increased at 21 and 28 days post-treatment. It is difficult to explain why these hormones increase at these particular time intervals but it is unlikely to be due to a direct effect of the compound, since it would have been metabolised and excreted within the first 48 to 72h post-treatment. Therefore it is more likely to be a consequence of mDNB treatment; the rise in IF inhibin levels 21 days post-treatment possibly reflects the appearance of particular germ cell types which modulate Sertoli cell inhibin secretion and which had degenerated and/or were absent up to this time. The increases in serum and IF testosterone levels in the absence of increases in serum LH may represent re-establishment of normal paracrine communication between Sertoli cells and Leydig cells. Without further work these hypotheses remain to be clarified.

A major problem in establishing the testicular effects of mDNB *in vivo* was the unexplained between animal variability, evident in testicular

histology of different animals at identical times after mDNB administration. In some animals the degree to which spermatogenesis was affected by mDNB was negligible and at 24h after treatment ranged from only minor Sertoli cell vacuolation with no major germ cell loss to complete disruption of spermatogenesis in all seminiferous tubules. This phenomenon of inter-animal variability has been observed by other investigators (Foster - personal communication). The variability was more apparent in hormonal levels between animals than in testicular histology. This probably reflects the fact that only 2 to 3 animals were utilised at each time interval for testicular histological examination while 6 to 7 animals were used to identify changes in hormonal levels post-treatment. In addition, because no serum or IF samples were removed from perfused animals it is impossible to correlate changes in hormonal profiles for individual animals to testicular histology per animal. If a decrease in testicular weight was used as an index of testicular toxicity, a degree of correlation between significant changes in hormonal profiles per animal and a decrease in testicular weight for the same animal were observed. Overall, however, these results were too inconsistent and unreliable to make direct comparisons for all animals. The reason for this variable effect is unknown, but could be explained by the route of administration of the compound and its pharmacokinetic properties. In order for a compound, which is administered orally, to enter the circulatory system it must be absorbed by the gut. Previous investigators have demonstrated the necessity for intestinal gut flora in the metabolism and absorption of nitro aromatic compounds (Parke, 1961; Facchini & Griffith, 1981). In germ-free rats pre-treated with antibiotics the testicular toxicity of mDNB was markedly enhanced, whilst the haematological effects were removed. Therefore it is possible that differences between animals in the intestinal gut flora and/or differences in the rate of absorption might alter the peak blood levels of mDNB, which in turn might account for the variability observed in these *in vivo* experiments. In addition, numerous investigators (Blackburn *et al*, 1988; Hess *et al*, 1988) have administered doses of mDNB at 48-50 mg/kg bodyweight: In these studies doses of 25 mg/kg bodyweight were used. Therefore, it is possible that, in some animals 25 mg/kg induces very little testicular toxicity whilst in other animals it causes severe testicular disruption.

Exposure of Sertoli cells in culture to mDNB resulted within 24h in changes similar to those observed *in vivo*. At the histological level Sertoli cell vacuolation was commonplace coupled with the degeneration and phagocytosis of different types of germ cells in culture at a number of doses, while the exfoliation of germ cells in co-culture was also observed, the latter being dose-

dependent. In addition, inhibin secretion by Sertoli cells in the absence or presence of germ cells has been used as a further index of Sertoli cell function in culture. Reproducible biphasic changes in inhibin secretion were observed following mDNB exposure in both culture systems. The present results confirm previous findings (Foster *et al*, 1986; 1987; Lloyd & Foster, 1987; Williams & Foster, 1988) in showing that mDNB induces specific perturbation of Sertoli cells *in vitro*, and that the spermatogenic disruption induced by mDNB *in vivo* is therefore probably a result of a direct effect on the Sertoli cell. The present results also explore the possibility that inhibin may be a useful marker of the toxic actions for compounds on the Sertoli cell.

The histological events following mDNB administration in culture and the exfoliation of viable germ cells (at most doses) are effects consistent with previous studies (Foster *et al*, 1986; 1987) and point to a direct effect of mDNB on the Sertoli cell. In particular, germ cell necrosis and the resultant phagocytosis of these germ cells were events which occurred in cultures exposed to 'toxicologically relevant' concentrations. Foster *et al* (1987) demonstrated that peak blood and testis concentrations of radiolabel following a single oral dose of 50 mg/kg $m[^{14}C]$-DNB to rats were 5 and $2x10^{-5}M$ respectively; a dose which produced a significant decrease in testis weight. Assuming a uniform distribution within the testis and a minimum of 90% of the radiolabel present was in the form of mDNB, then in this study, significant effects in culture were evident below the testicular concentrations of mDNB calculated from these *in vivo* studies. Furthermore, Foster *et al* (1987) demonstrated germ cell exfoliation and Sertoli cell vacuolation in culture at concentrations in excess of $5x10^{-6}M$ mDNB. In these studies significant germ cell exfoliation was evident at concentrations in excess of $1x10^{-6}M$ mDNB while Sertoli cell vacuolation was observed initially following the administration of $1x10^{-5}M$ mDNB. Taking this into account, the degeneration of germ cells observed *in vitro* 24h after mDNB administration may be similar to that observed *in vivo*, with pachytene spermatocytes degenerating 24h after the oral administration of mDNB to rats.

Previous studies have used a number of indices of Sertoli cell function to identify cellular perturbation induced by mDNB, these have included basal lactate and pyruvate secretion (Williams & Foster, 1988). Although these two parameters have been used to assess Sertoli cell function following nitrobenzene administration to cultures (see Chapter 4), they have not been used here. The present study has measured an index of Sertoli cell function not previously used to assess toxic action *in vitro*, namely inhibin secretion. Basal

inhibin secretion exhibited a reproducible alteration in response in a biphasic manner following exposure of co-cultures or Sertoli cell cultures to mDNB. Since this pattern of response was observed in both co-cultures and mono-cultures it is unlikely to be due to the exfoliation of selective germ cell type(s), a possibility that arises from the probable control of inhibin secretion by round and elongate spermatids (Pineau *et al*, 1990). Furthermore, in comparisons of inhibin secretion by Sertoli cells in the presence or absence of germ cells the elevated and sustained secretion of inhibin in the presence of germ cells is further evidence of germ cell modulation of Sertoli cell inhibin secretion. Significant rises in Sertoli cell inhibin secretion were observed in the presence of mDNB at toxicologically relevant concentrations. The only exceptions to this were at 1×10^{-4}M mDNB in both culture systems (a dose employed as a positive control in previous studies using nitrobenzene - see Chapter 4) and 5×10^{-3}M mDNB in co-culture. Williams and Foster (1988) also reported significant dose-dependent increases in the secretion of lactate and pyruvate from Sertoli cells cultured in the presence of mDNB at concentrations similar to those reported here which resulted in stimulation of inhibin secretion. Studies on the effects of mDNB *in vivo* suggest that increases in inhibin secretion were evident 24 and 72h after mDNB administration as measured in testicular IF. The increase in inhibin secretion evident in culture following mDNB administration may reflect increases *in vivo* observed at 24h post-treatment. These increases may reflect Sertoli cell perturbation and germ cell degeneration *in vivo* observed by histological observation 24h post-treatment.

In summary, the present studies confirm previous investigations which demonstrate that mDNB is a testicular toxicant. Furthermore, confirmation of its site of action was obtained *in vitro* in this and previous studies pinpointing the Sertoli cell as the target for mDNB. The present study is also one of the first to evaluate the potential of inhibin as a marker for toxicant action on the Sertoli cell. Further work is required to assess the potential of inhibin as a Sertoli cell marker both *in vivo* and *in vitro* to add to the known and currently used markers of testicular function.

CHAPTER 4.

NITROBENZENE.

4.1. INTRODUCTION.

Nitrobenzene (NB) is an oily liquid, yellow in colour, with a bitter almond odour. One of the earliest applications of NB was as a perfume and soap scent called 'essence of mirbane'. First synthesised by Mitscherligh in 1834, NB is today an important chemical industrial intermediate employed primarily (98%) as an inexpensive oxidising agent and in the synthesis of aniline as a by-product. It is also used in the production of dyes, solvents, propellants, medicinal and agro-chemicals, and in the manufacture of numerous aromatic nitro compounds including *meta* - dinitrobenzene, (mDNB see Chapter 3) and aromatic amines. In 1978, annual worldwide production of NB exceeded half a million tons with estimates of 8 to 20,000 tons released annually into the environment arising from the loss of NB through emission or release during manufacture or use, (Dorigan and Hushon, 1976; Klapproth, 1979). The amount released is estimated to be 1.5% of the total U.S. production (U.S.A.-E.P.A., 1981), causing the contamination of three principle environmental systems - atmosphere, hydrosphere and agrosphere.

Since the begining of the twentieth century exposure to NB has been known to result in organ/tissue damage and over the last few decades has been associated with pathophysiological effects in man and animals. NB poisoning in man has been recognised since at least 1856 (Chandler, 1919). The majority of reported cases occurred following acute exposure by inhalation, or by dermal or oral routes (Gregory, 1906; Hamilton, 1919; Harrison, 1977). NB is absorbed readily through human skin and lungs. Data suggests that "adequate protection from skin absorption of NB is not afforded by street clothes" (Piotrowski, 1967). Methaemaglobinaemia is the predominant acute effect of NB reported in humans and laboratory animals with neurotoxicity and hepatotoxicity also evident from notes of human case histories after exposure. Blood disorders have also been reported during the stages of acute and chronic NB poisoning. Nevertheless, considerable variation exists between the absorbed dose of NB and the severity of response in man. With the exception of blood disorders, very little evidence of chronic toxicity of NB in humans is available, but case studies of workers exposed acutely to NB suggest that the liver, kidneys, spleen and possibly the testes may be target organs for NB after chronic exposure. Indeed, cases have been published (1950's) which refer to the use of NB as an abortifacient. Fortunately, although after numerous fatalities (50% mortality rate reported in 24 cases), its use was discontinued because of maternal toxicity.

Although there is extensive literature devoted to acute NB poisoning, the vast majority of reports deal with clinical cases and are of little value in relating the extent of exposure to toxicity. They deal with end-point evaluations of acute toxicity often identified at post mortem without knowing the conditions of exposure, dose, physiology etc up to the death of the misfortunate individuals. Obviously a greater understanding of NB toxicity, toxic doses and organs involved are required in order to set standards, guidelines and regulations pertaining to NB exposure and its hazard assessment in man and to be able to relate this to the clinical situation.

The LD_{50} for orally administered NB in rats is 640 mg/kg bodyweight (Fairchild, 1977). Beachamp *et al* (1982), have compiled a comprehensive review of the literature on NB toxicity. In this review, the potential target organs of toxicity after acute and chronic NB administration in rats are the blood (general pathological disorders and methaemaglobinaemia production), central and peripheral nervous system (neuronal degeneration), and hepatotoxicity with enlarged liver and altered serum chemistry. Miscellaneous effects include adrenal gland degeneration, pulmonary lesions, possible carcinogenic and teratogenic effects, and toxicity to the reproductive organs.

Studies concerned with the potential reproductive effects of NB administration to male rats were first initiated by Bond and co-workers in 1981. These studies demonstrated that in Fischer 344 rats (F-344), exposed to acute oral doses of NB (50 to 450 mg/kg bodyweight), degenerative testicular and hepatic lesions were formed. Testicular lesions were confined to the seminiferous tubule and consisted of dose- and time- dependent degeneration of primary and secondary spermatocytes followed by necrosis of spermatogenic cells at days 2 and 3 after 450 and 300 mg NB/kg bodyweight respectively. Subsequently, multinucleated giant cells appeared in the place of the necrotic spermatogenic cells and there was a decrease in spermatozoa in the seminiferous tubule with resultant necrotic debris and a decrease in sperm number in the epididymis as early as 3 days after NB administration. There were no apparent effects on the epididymal epithelium or on spermatogonia in the testis. These degenerative changes in testicular and hepatic architecture and function were not caused by methaemaglobin formation and were probably due to a direct effect of NB or its metabolites. Testicular lesions were evident only at doses of 200 mg/kg and above and were consistently produced at 300 mg/kg; they were not observed at doses below 75 and 160 mg/kg. Bond concluded that, "testicular damage resulting from NB exposure may be reversible, however a long time may be required before complete reversibility of the testicular lesion may occur".

Taking Bond's initiative, Levin *et al* (1983) investigated the reversibility of NB induced testicular lesions in F-344 rats using sperm output as an index of spermatogenic disruption equated with histological examination of testes from rats treated identically in parallel experiments. Regular sequential measurements of sperm output were made using a non-invasive technique: Surgical anastamosis of the vas deferens to the urinary bladder was performed such that daily urine samples could be collected to develop a profile of testicular damage per animal. Observations indicated that the testicular damage resulting from a single oral dose of NB (300 mg/kg) was reversible to the extent that sperm output was restored to near normal. A lag of 26 to 30 days between treatment and reduced sperm output was observed. Between 30 and 37 days sperm were not present in the urine of treated rats, but thereafter there was a gradual recovery to 78% of control values by 76 to 100 days post-treatment. Complete recovery was precluded however by a limited number (approximately 10%) of tubules which were severely disrupted, failed to regenerate, or were abnormally developed. Levin *et al* (1983) and evidence published by Dodd and co-workers (Dodd *et al*, 1987), discussed later, concluded that restoration of spermatogenesis occurred after cessation of exposure to NB and this suggests that undifferentiated germ cells and spermatogonia initiating differentiation were resistant to toxicity (Amann, 1982).

Since NB is a mildly volatile liquid (0.28 mm Hg at 25°C), the potential for NB to induce toxicity, be it testicular or otherwise, is probably manifested through inhalation as the route of absorption. Initial work which exposed F-344 and CD rats or B6C3F1 mice to atmospheres of NB produced bilateral degeneration of the seminiferous epithelium and a reduction or absence of sperm in the epididymis of rats, although the mice had no testicular lesions. The severity of these lesions were dependent upon strain and species differences (Medinsky & Irons, 1982; Hamm *et al*, 1984).

Further investigations centred on the metabolism of NB to identify if the parent compound or a metabolite were responsibile for the demonstrated marked strain and species difference in NB toxicity (Rickert *et al*, 1983). In rats and mice urinary elimination of NB was the most important clearance route with NB metabolites peaking at 6 to 24 hours after administration of ^{14}C-NB (225mg/kg), regardless of the route of entry (Rickert *et al*, 1983). A number of these metabolites were isolated from the urine of rats and mice, of those metabolites which could be identified nitrophenols and aminophenols were the main constituents, of which 50% of the single oral dose excreted in the urine were conjugates of these metabolites. From those metabolites which could be

identified there were no strain differences in the composition of rat urine and only one other metabolite was isolated in mouse urine. There were, however, substantial strain and species differences in the elimination of unidentified metabolites of NB. Rickert *et al* (1983) concluded that slow absorption of NB from the intestine did not account for the delay in attaining peak excretion rates, and that NB was therefore metabolised slowly in both rats and mice. In addition to species differences, the mechanism(s) of NB-induced toxicity are complicated further by the influence of diet (Goldstein *et al*, 1984). The gut microflora is an important site for reductive metabolism because of its anaerobic environment and high activity of nitroreductase, which is known to be required for the induction of methaemaglobinaemia in NB- and mDNB-treated rodents (Reddy *et al*, 1976; Facchini & Griffiths, 1981 and Chapter 3). This is only relevant in studies involving the oral administration of compounds. The final reduction products excreted in the urine are the result of gut flora activity as urinary metabolites of NB produced by rodents pretreated with antibiotics to render them germ-free are different from those produced in rats with a normal gut flora. Thus NB metabolism *in vivo* is the result of a complex series of metabolic processes of both bacterial and mammalian origin (Levin & Dent, 1982).

Recently, both teratogenic and reproductive/fertility evaluations in rats have been performed following inhalation of NB (Tyl *et al*, 1987; Dodd *et al*, 1987). In fertility evaluations a two generation reproduction study was performed following chronic exposure of CD rats to NB vapour. The results showed that exposure to NB reduced the fertility of the F_0 male rats. However, these effects only occurred at a dose of 40 ppm for 6h/day for 5days/week for 10 weeks (the highest dose used in the study), and were manifest as a decrease in the fertility index. The subsequent mature F_1 male generation sired from the F_0 male generation after a period of recovery also showed similar effects after exposure to NB and, as with the F_0 male generation, a time-dependent reversibility upon removal of NB (as judged by an increase in the fertility index) was observed. Therefore, in instances in which NB-induced testicular disruption was produced (both in this study and that of Levin *et al*, 1983), a longer recovery period would likely produce a greater incidence of restoration of spermatogenesis and, subsequently, a higher fertility index for exposed F_0 and F_1 generations. No *in utero* effects of NB on testicular organogenesis were observed. Further studies established no developmental toxicity to other organs or teratogenicity in CD rats exposed to NB vapour during organogenesis, although some maternal toxicity was detected at 10 and 40 ppm.

In summary, *in vivo* experiments in rodents (Bond *et al*, 1981, Medinsky & Irons, 1982; Levin *et al*, 1983; Hamm *et al*, 1984; Dodd *et al*, 1987 and Tyl *et al*, 1987) have established that; a) NB is a toxicant with the testes as a target organ producing dose-dependent testicular disruption, b) the testicular effects are manifested via three routes of administration, namely oral, inhalation and possibly dermal, c) the rat is a sensitive species to NB-induced testicular effects, although probably not as sensitive to testicular toxicants as man (discussed later), d) the effects to the testes after acute exposure to NB are time-dependent and reversible and, e) NB has effects on maternal reproductive performance but has no *in utero* organogenic or teratogenic potential in rats. However from the male fertility veiwpoint, questions still need to be addressed.

Publications to date have addressed the testicular toxicity of NB at a relatively superficial level. The cellular mechanism underlying this toxicity remains to be identified. Previous investigators have postulated that NB has a direct effect on the Sertoli cell but little evidence exists to substantiate this claim. Investigations into the initial site and mechanism of action of spermatogenic disruption induced by NB *in vivo* have yet to be fully clarified or understood. There are no studies on the potential testicular toxicity of NB *in vitro*. Furthermore, the pharmacokinetic properties of NB in the testis, its absorption, distribution and elimination are unknown. It is therefore unknown whether the parent compound distributes within the testes or if metabolite(s) of NB produced by other organs or within testicular cells are the cause of spermatogenic disruption.

The introductory review of spermatogenesis in Chapter 1 centred on the endocrine and paracrine control. There are known feedback loops from the testes to the pituitary and hypothalamus, others as yet unidentifed may also exist. These feedback hormones are secreted by testicular cells and, therefore, cellular perturbation by toxicants directed towards these cells may induce changes in the secretion of these hormones which could be evident in the serum or interstitial fluid (IF) of NB-treated animals. If these changes do exist, they may be time-dependent and as the lesion progresses, serum hormone levels may change in response to this progression. Secretion of paracrine factors into IF may also be altered acutely (i.e. directly after toxicant administration), or chronically. Therefore, both serum and intratesticular hormonal profiles after NB treatment may allow an insight into biochemical changes taking place throughout the progression of a lesion within the seminiferous epithelium. Histological observations taken at identical time intervals could also give a greater insight into the precise nature of the lesion, identify which cells are

initially affected, what effect this has on neighbouring cells and how the NB lesion progresses in a time-dependent manner through the spermatogenic cycle. These aspects of NB-induced testicular disruption have never been studied in any great detail *in vivo*.

Studying NB-induced testicular disruption *in vitro* using Sertoli cell cultures may also aid in our understanding of the biochemical mechanisms behind cellular perturbation (e.g. if the Sertoli cell is the target for NB) and the results of these effects on spermatogenesis and cellular function. Using this culture system, predictions can possibly be made about the events *in vivo* which result in spermatogenic dysfunction. Indices of cellular function including lactate, pyruvate and inhibin may aid in our assessment of perturbation. Therefore any studies performed *in vivo* require further investigation *in vitro* using a suitable culture system. The aim of the present study was to examine the testicular toxicity of NB to identify if the Sertoli cell was the target, and to examine hormonal profiles *in vivo* as an index of events occurring *in vivo* and equate these to temporal histological changes in addition to investigating further the effects of NB on the Sertoli cell *in vitro*.

4.2. METHODS.

4.2. Methods for assessment of the effects of NB *in vivo*.

4.2.1. Reagents.

NB was obtained from Sigma (Poole, Dorset, U.K). Analysis by gas chromatography/mass spectrometry for composition before use found it to be 99% pure. The vehicle for NB administration was corn oil (Mazola) obtained from a supermarket. The corn oil was stored under nitrogen gas to prevent its oxidation.

4.2.2. Animals.

Rats were of the Sprague Dawley derived strain bred in the Centre for Reproductive Biology in Edinburgh, U.K.

4.2.3. Experimental Protocol.

Adult male rats (80-130 days old) were randomly allocated 8/cage and maintained under normal animal house conditions. Food and water were available *ad libitum*.

At the beginning of the experiment (time 0) rats were administered NB by oral gavage at a single dose of 300 mg/kg bodyweight in corn oil (1ml/kg). Control groups were administered vehicle alone (1ml/kg). Animals were weighed daily for the first 14 days post-treatment in order to detect any significant decrease in body weight which may have indicated overt toxicity. At selected time intervals after dosing rats were subjected to one of the following procedures;

a) 2 to 3 rats/group were anaesthetised and perfusion-fixed for histological preservation and later examination of the testes and epididymides (see section 2.4.1.).

b) 5 to 6 rats/group were killed by CO_2 inhalation followed by cervical dislocation, the testes were removed and weighed, blood was collected immediately after death for hormone measurement (see section 2.3.) and testicular interstitial fluid (IF) was drip collected overnight (see section 2.2.1.) for hormone measurements. Serum hormones measured were FSH, LH and testosterone whilst testosterone and inhibin levels were measured IF.

The successive time intervals at which groups of NB-treated animals were killed were 1, 3, 7, 14, 21, 28, 35, 42 or 70 days post-treatment whilst control groups were killed at 3, 14, 35 or 70 days post-treatment.

4.2.4. Method for evaluating a change in frequency of occurrence of the stages of the spermatogenic cycle.

Following identification of the initial NB-induced lesions in the testes of treated rats and the progression of these lesions at subsequent time points after dosing, it appeared that there was a change in the frequency of occurrence of certain stages of the spermatogenic cycle in comparison to controls. To test if this was the case, at each time point 100 transverse sections of seminiferous tubule per animal were examined histologically and staged according to the classification of Leblond & Clermont, 1952 (see Fig 1.2). Both treatment and control animals were examined, utilising numerous cross sections of testicular tissue taken from both testes of each animal per group. Certain stages of the spermatogenic cycle were then grouped together. These groups were I-IV, V-VI, VII-VIII, IX-XI and XII-XIV. Results were then expressed as a percentage frequency of occurrence of each group of stages per treated rat for each time point in comparison to the stage frequency in control rats. Since the latter remained constant in the control groups sampled throughout the

experiment, control data were pooled and therefore only one sequence of control stages is illustrated in the graphs (see Fig 4.6). Data were analysed by two way Analysis of Variance with replication. Tubule cross sections which, because of treatment, had far too few germ cells to permit confident histological classification or which contained only Sertoli cells were observed at certain time points after treatment and were classified as Sertoli cell only tubules. These unclassified and/or Sertoli cell only tubules contributed a varying percentage of the total number of tubules staged at each time point, but were not observed histologically until 14 days post-treatment and, at this time point, their frequency, expressed as a percentage of the total tubules counted was very small $(4.8 \pm 2.1\%$; mean $\pm$ SD). The percentage of Sertoli cell only tubules increased at 21 and 28 days post-treatment $(6.3 \pm 1.3\%$ and $7.5 \pm 2.0\%$ respectively), and remained approximately constant at the remaining time points. However the change in the frequency of occurrence of stages of the spermatogenic cycle was observed much earlier (at 3 and 7 days post-treatment). Therefore, it is unlikely that discounting these unclassified and/or Sertoli cell only tubules from the cross sections counted and staged at each time point would bias the results. Unclassified and/or Sertoli cell only tubules were not identified until after the change in the frequency of occurrence of stages had occurred and therefore could not have contributed to the frequency changes observed before 14 days or those observed up to 70 days post-treatment.

4.3. Methods for assessment of the effects of NB *in vitro*.

4.3.1. Reagents.

For the purity of NB see section 4.2.1. and for mDNB see section 3.2.1. Porcine pituitary FSH was obtained from Sigma (Poole, Dorset, U.K). All culture materials were obtained from Flow Laboratories (Irvine, Scotland, U.K.) or Gibco (Paisley, Scotland, U.K). All other biochemical reagents were obtained from Sigma, B.D.H. (Poole, Dorset, UK) or Boehringer Mannheim (Lewes, East Sussex, U.K.) and were of the highest purity available.

4.3.2. Animals.

Rats were of the Alpk:AP (Wistar derived) strain from the I.C.I. Animal Breeding Unit, Alderley Park, Macclesfield, Cheshire, U.K.

4.3.3. Experimental Protocol.

4.3.3.1. Preparation of Testicular cell cultures.

Cultures were prepared using a modification of the method of Gray & Beamand (1984) while Sertoli cell only cultures were prepared from co-cultures using the method of Galdieri *et al* (1981) (see section 2.5.1.).

4.3.3.2. Treatment of Cultures.

Toxicants were administered to cultures 72h after the plating of cells. NB was administered at a range of concentrations of between $5 \times 10^{-8} M$ and $5 \times 10^{-3} M$. mDNB at a previously identified toxic concentration of $1 \times 10^{-4} M$ was used as a positive control for comparison (see Chapter 3). Both toxicants were administered over a 24h incubation period applied as solutions in dimethylsulphoxide (DMSO), final concentration 0.3% v/v, while control cultures received an equal volume of this vehicle. Treatments caused no appreciable alteration of culture medium pH. All experiments were performed at least three times, in each case with a minimum of six replicate cultures for each dose and treatment group.

Porcine FSH (0.021U/well) was applied directly to the cultures at the time of toxicant addition. Previous data using these culture preparations (Williams & Foster, 1988) had demonstrated that this concentration of FSH resulted in maximal stimulation of both lactate and pyruvate secretion.

4.3.3.3. Parameters measured.

Medium was removed from cultures 24h after toxicant administration for the measurement of inhibin (see section 2.3.3.), lactate and pyruvate (see section 2.5.4.), and for quantification of germ cell exfoliation (see section 2.5.8.). The remaining cells in each well were used for protein estimation (see section 2.5.8.). Cultures containing cover slips were stained using haematoxylin and Papanacolaou's stain for histological examination (see section 2.5.2.).

4.4. RESULTS.

4.4.1. Effect of NB on testicular weight.

Treatment with NB induced a decrease in testicular weight within 3 days (Fig 4.1) in comparison with an average control testicular weight made up of the sum of the four control groups. This decrease reached a nadir at 35 days post-treatment. Between 35 and 70 days after NB-treatment, testicular weight recovered towards control values but was still reduced significantly in comparison to the testicular weight average for the control groups.

4.4.2. Effect of NB on serum levels of FSH.

Within 24h of a single oral dose of NB, serum FSH levels were raised significantly (Fig 4.2). Levels continued to rise at 3 days and by 7 days were at their highest levels. At 14 days post-treatment serum FSH levels in treated rats had begun to decline but remained significantly higher than the overall control level. By 21 days post-treatment serum FSH levels were not significantly different from controls and remained so up to 70 days post-treatment.

4.4.3. Effects of NB on serum levels of LH.

In NB-treated rats serum LH levels did not change significantly except at 14 days post-treatment, but overall, levels were higher in NB-treated rats over the first 14 days post-treatment (Fig 4.2)

4.4.4. Effect of NB on interstitial fluid (IF) levels of inhibin

Immunoactive levels of inhibin increased significantly in testicular IF collected from NB-treated rats after the first 24h and remained elevated to an equivalent extent at 3, 7 and 14 days post-treatment (Fig 4.3). Between 14 and 21 days, IF levels of inhibin in NB-treated rats began to decline such that by 21 days, the levels were not significantly different from inhibin levels in testicular IF in the overall control group (i.e. comprising the four control groups shown in Fig 4.3). IF levels of inhibin did not vary from those of controls over the remaining time points up to 70 days post-treatment. The serum levels of inhibin were not measured for reasons given in the Discussion (section 4.5).

4.4.5. Effect of NB on serum and IF levels of testosterone.

The levels of testosterone in IF and serum were both extremely variable, and this was equally evident in control and treated animals (Fig 4.4).

Fig 4.1. Effect of a single oral dose of nitrobenzene (300 mg/kg bodyweight) (■) or vehicle alone (1ml/kg) (▨) on testicular weight in groups of rats at intervals after dosing. Values are means ± SD for 6 rats (*P<0.01, **P<0.001, in comparison to an overall control value [dotted line] made up of the average of the four control groups).

Fig 4.2. Effect of a single oral dose of nitrobenzene (300 mg/kg bodyweight) (■) or vehicle alone (1ml/kg) (▨) on serum levels of FSH (a) and LH (b) in groups of rats at intervals after dosing. Values are means ± SD for 6 rats (*P<0.01, **P<0.001, in comparison to an overall control value [dotted line] made up of the average of the four control groups).
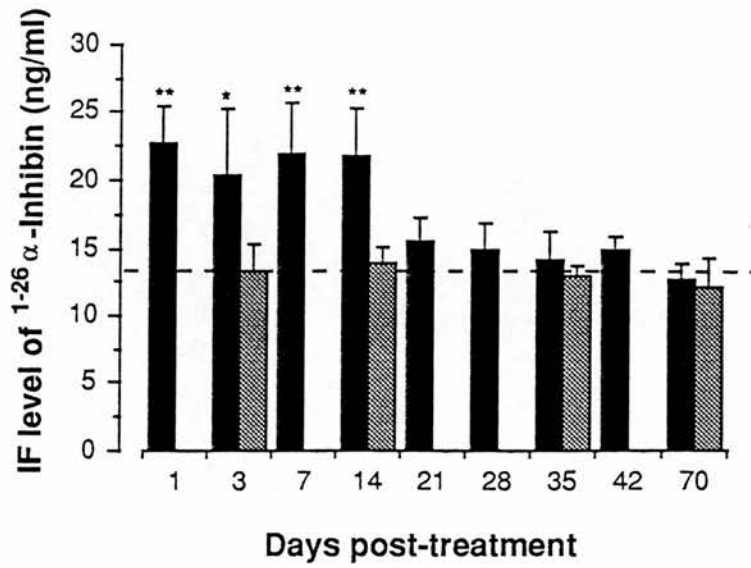
Fig 4.3. Effect of a single oral dose of nitrobenzene (300 mg/kg bodyweight) (■) or vehicle alone (1ml/kg) (▨) on immunoactive inhibin levels in testicular interstitial fluid (IF) from groups of rats at intervals after dosing. Values are means ± SD for 6 rats (*P<0.01, **P<0.001, in comparison to an overall control value [dotted line] made up of the average of the four control groups).
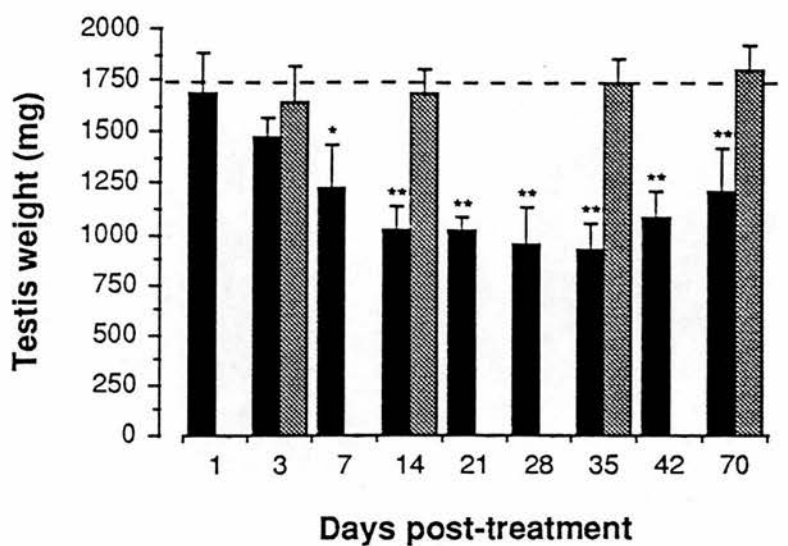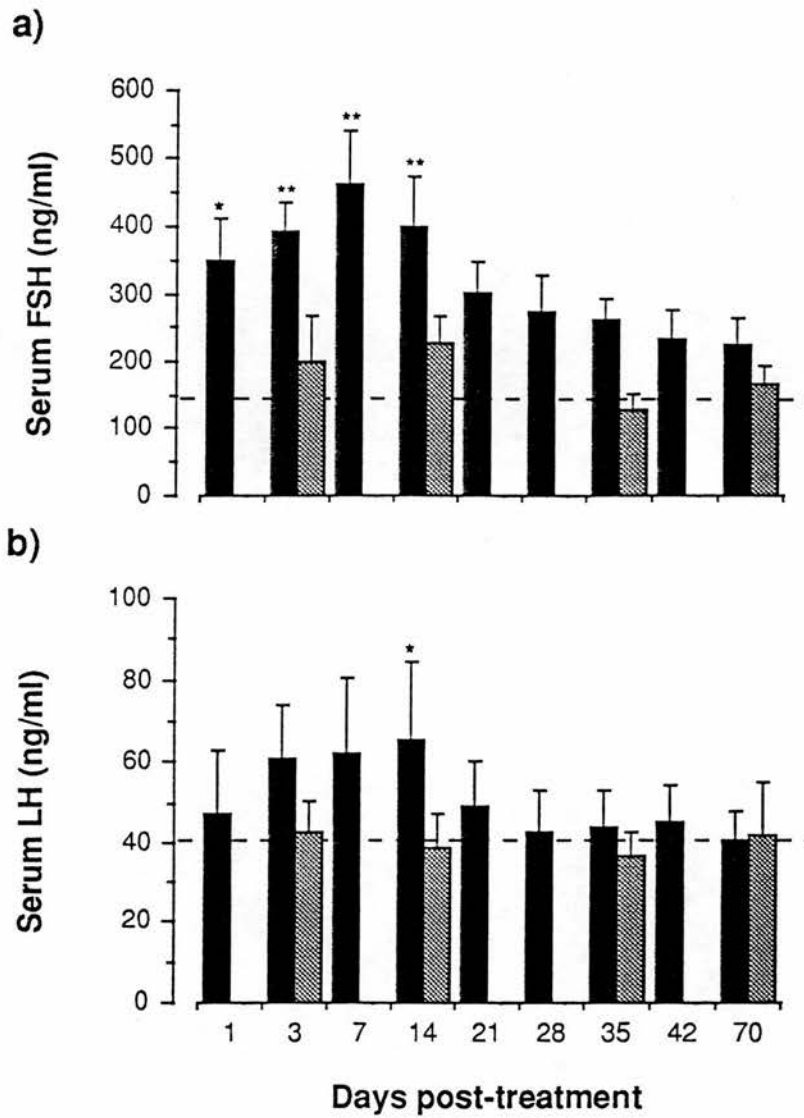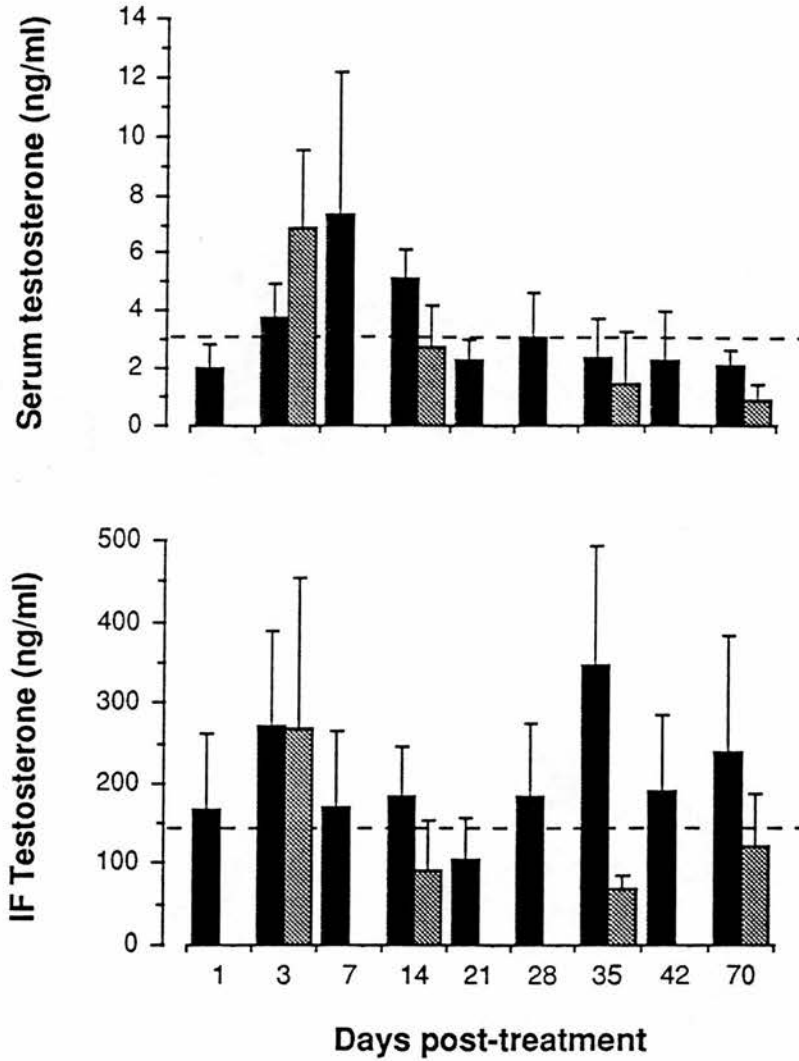
Fig 4.4. Effect of a single oral dose of nitrobenzene (300 mg/kg bodyweight) (■) or vehicle alone (1ml/kg) (▨) on serum (a) and testicular interstitial fluid (IF) (b) levels of testosterone in groups of rats at intervals after dosing. Values are means ± SD for 6 rats. No significant differences (P>0.05) were observed.

No significant effect of NB on IF and serum levels of testosterone was observed and no evidence for any pattern to the results could be observed.

4.4.6. Effect of NB on testicular morphology.

Testicular tissue taken from control rats in the 3, 14, 35 or 70 day groups exhibited normal histology with no germ cell exfoliation or abnormal cellular necrosis (see plate 4.1 a,b and c), and with apparently normal kinetics of spermatogenesis as judged by the frequency of the different stages when compared with published data. (Leblond & Clermont, 1952).

A single administration of NB caused rapid and extensive disruption of spermatogenesis in all treated rats. Within 24h of exposure to NB vacuolation of Sertoli cells and the epithelium had occurred (see plate 4.2. a), the integrity of the epithelium had begun to break down (plate 4.2. b) and many pachytene spermatocytes were absent or appeared pyknotic (plate 4.2. c). These changes appeared to be stage-specific, with stages VII to XII being more affected than stages I to VI. The former contained large vacuoles and numerous degenerating pachytene spermatocytes (plate 4.2. c), particularly in stages VII to VIII (plate 4.2. d). Pachytene spermatocytes in stages VII to XII also appeared smaller than their control counterparts at these stages, however detailed morphometry is required to confirm this possibility. Stages I to IV also appeared damaged after NB-treatment (plate 4.3. a) with widespread degeneration of pachytene spermatocytes. Furthermore the nuclei of round spermatids in stages I to VI appeared diffuse in comparison to controls, and the chromatin within the nuclei was beginning to fragment at 3 days post-treatment (plate 4.3. a). Also at this time point in a number of tubule cross sections at stages VII to XIV, pachytene spermatocytes were absent or observably decreased in number in comparison to identical stages in control cross sections. It is assumed that these pachytene spermatocytes were either sloughed into the lumen, phagocytosed by Sertoli cells (these events were not observed by histological examination) or had coalesced to form giant bodies (plate 4.3.). There were also large vacuoles in the epithelium at 3 days post-treatment (plate 4.3. b) which perhaps represents an indication of phagocytosis of individual pachytene spermatocytes or giant bodies by Sertoli cells. These vacuoles were not intracellular to germ cells and were not artifacts as they were not associated with any sign of shrinkage or swelling around the base of the Sertoli cell (see also plate 4.1. a). They have therefore been classified as extracellular vacuoles.

Within 72h of NB exposure the nuclei of round spermatids appeared diffuse and abnormal in structure (plate 4.3. c) in comparison to
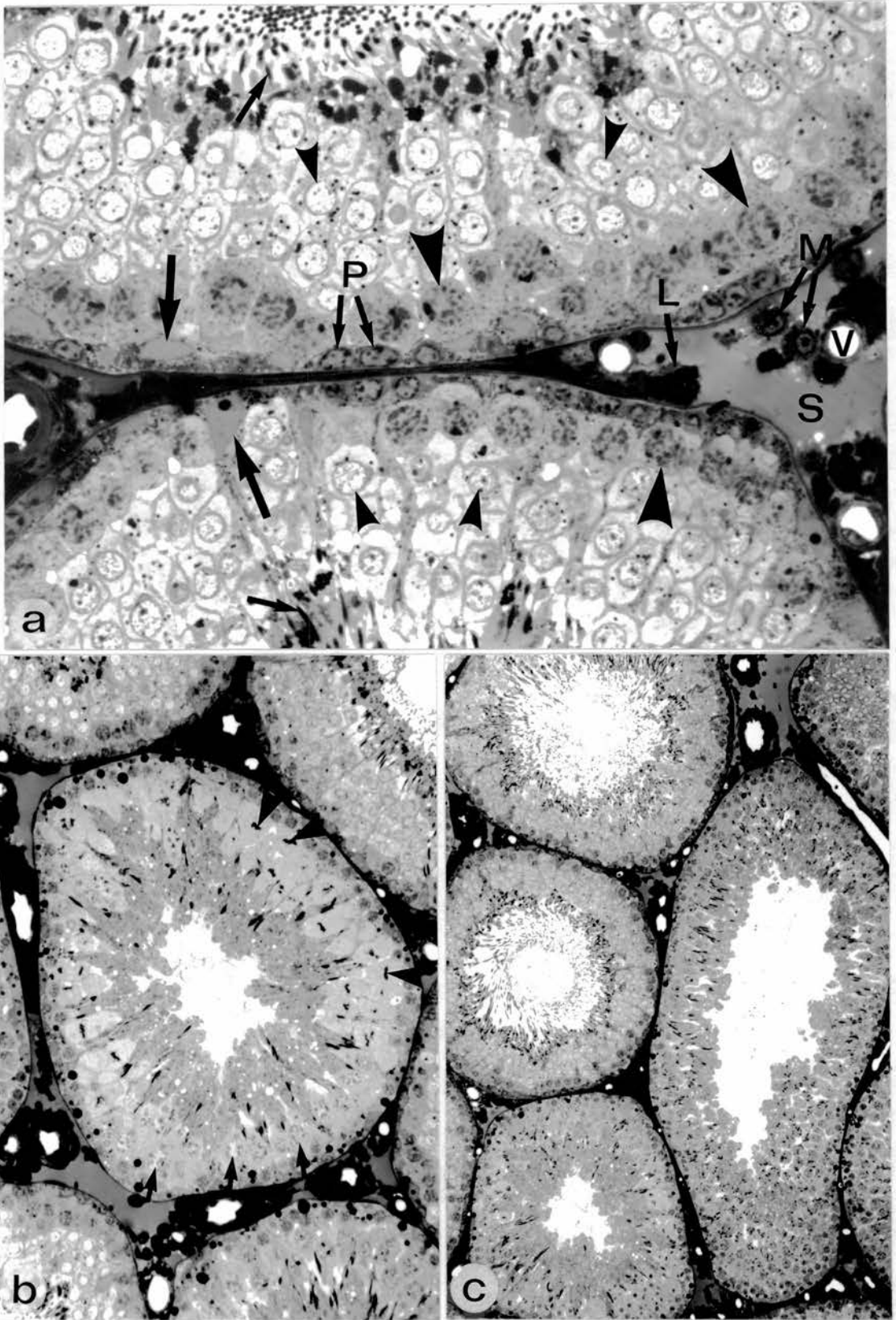
Plate 4.1. Seminiferous tubules from control rats illustrating (a) high power view of epithelium showing
Sertoli cell nuclei (large arrows) pachytene spermatocytes (large arrowheads) round spermatids (small
arrowheads) and the heads of elongate spermatids (small arrows). Note the formation of residual bodies in
the upper stage VIII tubule. Compare also the preleptene spermatocytes (P) and B spermatogonia (B).
The interstitial space (S), blood vessels (V), Leydig cells (L) and macrophage (M) are also shown (x400);
(b) section through a stage XIV tubule, note the large diplotene spermatocytes (arrows) while others are
going through metaphase with chromosomes visible (arrowheads) (x190). Compare with plate 4.4. c;
(c) low power view through a number of tubules (x75).

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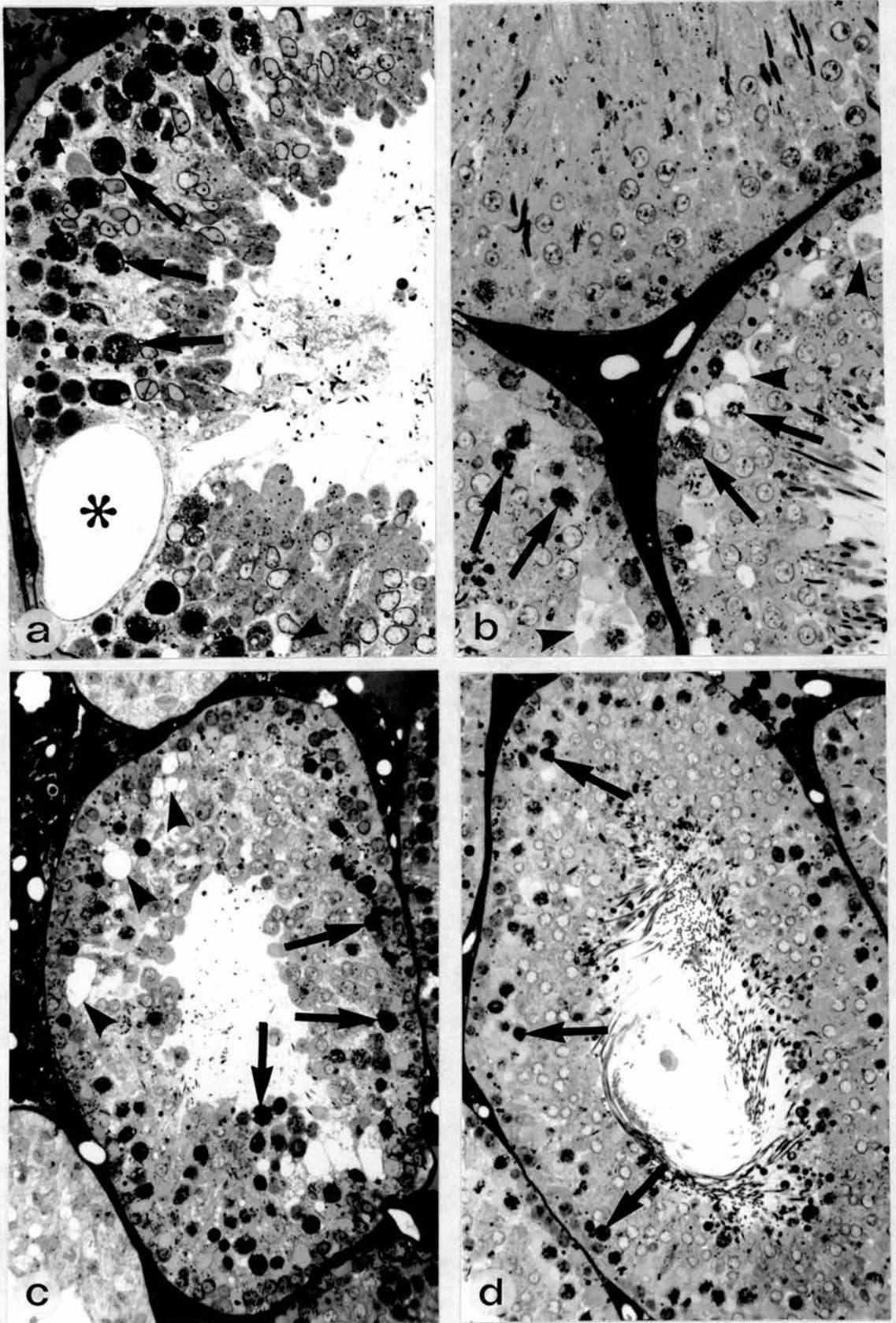
Plate 4.2. Status of spermatogenesis 24h after a single oral dose of NB illustrating (a) vacuolation of the epithelium (asterick) of a stage IX tubules in combination with pyknotic pachytene spermatocytes (arrows) (x300): (b) section through three adjacent seminiferous tubules showing degenerating pachytene spermatocytes (arrows) and vacuolation (arrowheads) within the epithelium of two stage VII tubules whilst the (upper) stage IV tubule appears qualitatively normal (x220): (c) stage VIII seminiferous tubule containing numerous pyknotic pachytene spermatocytes (arrows) and a vacuolated epithelium (arrowheads) (x220): (d) early stage VIII seminiferous tubule containing degenerating pachytene spermatocytes (arrows) (x190).
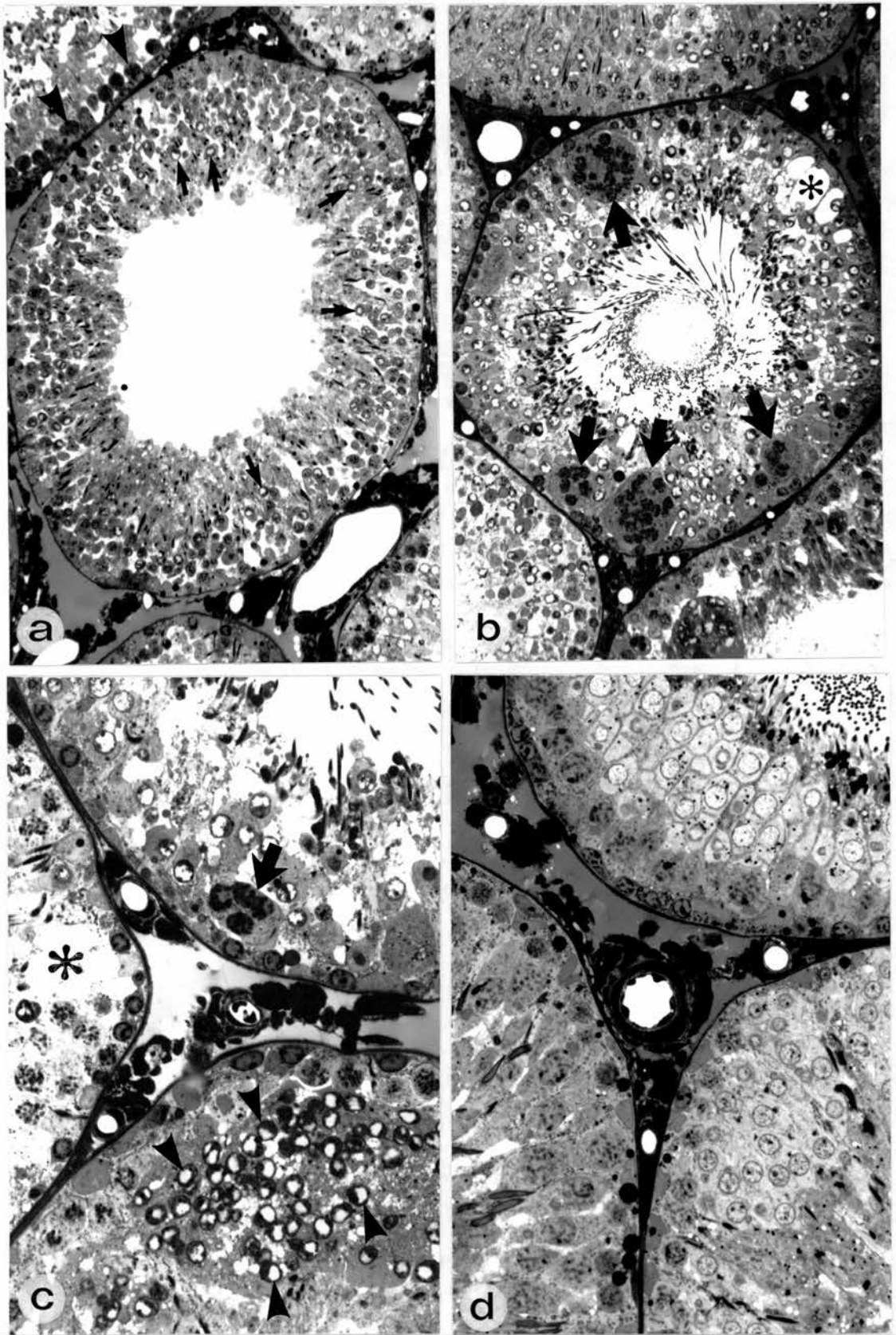
Plate 4.3. Seminiferous epithelium 72h after a single oral dose of NB illustrating (a) shrinkage of the nuclei and degenerative appearance of round spermatids (arrows) and degenerating pachytene spermatocytes in another tubule (arrowheads-top) (x190): (b) giant bodies (arrows) formed from the coalescence of pachytene spermatocytes and a large vacuole in the epithelium (asterick) (x190); (c) section through three adjacent seminiferous tubules showing round spermatids with abnormal nuclei (arrowheads), a giant body consisting of pachytene spermatocytes (arrow) and a large vacuole within the epithelium (astericks) (x300): (d) seminiferous epithelium from control animal showing normal appearance of pachytene spermtocytes (arrows) and round spermatids (arrowheads) within the epithelium (x300).

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control sections (plate 4.3. d). In NB-treated rats, the round spermatids were either sloughed into the lumen, phagocytosed by Sertoli cells or formed giant bodies in a similar manner to pachytene spermatocytes observed 1 day after treatment (plate 4.4. a and 4.4. b). The giant bodies formed from pachytene spermatocytes appeared distinctly different from those formed from round spermatids under histological examination (see plates 4.3. b, c and 4.4. a). In all sections examined containing giant bodies, each consisted of round spermatids or pachytene spermatids but in no giant body could pachytene spermatids and round spermatids be observed together. Stages of the spermatogenic cycle not containing round spermatids (VIII to XIV) exhibited loss or degeneration of pachytene and diplotene spermatocytes (plate 4.4. c). Elongating spermatids were still present at all the appropriate stages of the spermatogenic cycle and appeared morphologically normal in some stages (plate 4.4. d - stages VI to VII) while in others they appeared morphologically abnormal (plate 4.4. d - stage X) in comparison to respective stages in control sections.

At 7 days after NB exposure two distinctly different populations of seminiferous tubules were observed (plate 4.5. a). These differed in the degree of severity of lesions within the seminiferous epithelium (plate 4.5. b). A percentage of tubules had a relatively intact epithelium while the remainder were severely vacuolated (plate 4.6. a). The former possessed pachytene spermatocytes, which had developed from leptotene spermatocytes at the time of NB exposure. These pachytene spermatocytes appeared to be in excess of normal numbers, although detailed morphometry would be necessary to determine if this effect was real or due to compression of the epithelium (plate 4.6. b). The remaining tubules were vacuolated and contained a number of pachytene spermatocytes which appeared histologically normal despite gross damage within the epithelium and the loss or reduction in the normal complement of germ cells at these stages (plate 4.6. a). In both types of tubules round spermatids were completely absent, irrespective of the stage that the tubule was at, while elongate spermatids were either absent or appeared morphologically abnormal. Since the process of elongate spermatid maturation and spermiation of step 19 elongate spermatids takes in excess of 310h (Fig 4.5.), and since morphologically normal elongate spermatids were observed at 3 days post-treatment in a number of tubules (plate 4.4. d), then during the 96h interval between 3 and 7 days, it is presumed that elongate spermatids had either been phagocytosed by Sertoli cells or had been released prematurely into the lumen. Histological evidence for the former was apparent 3 days post-treatment
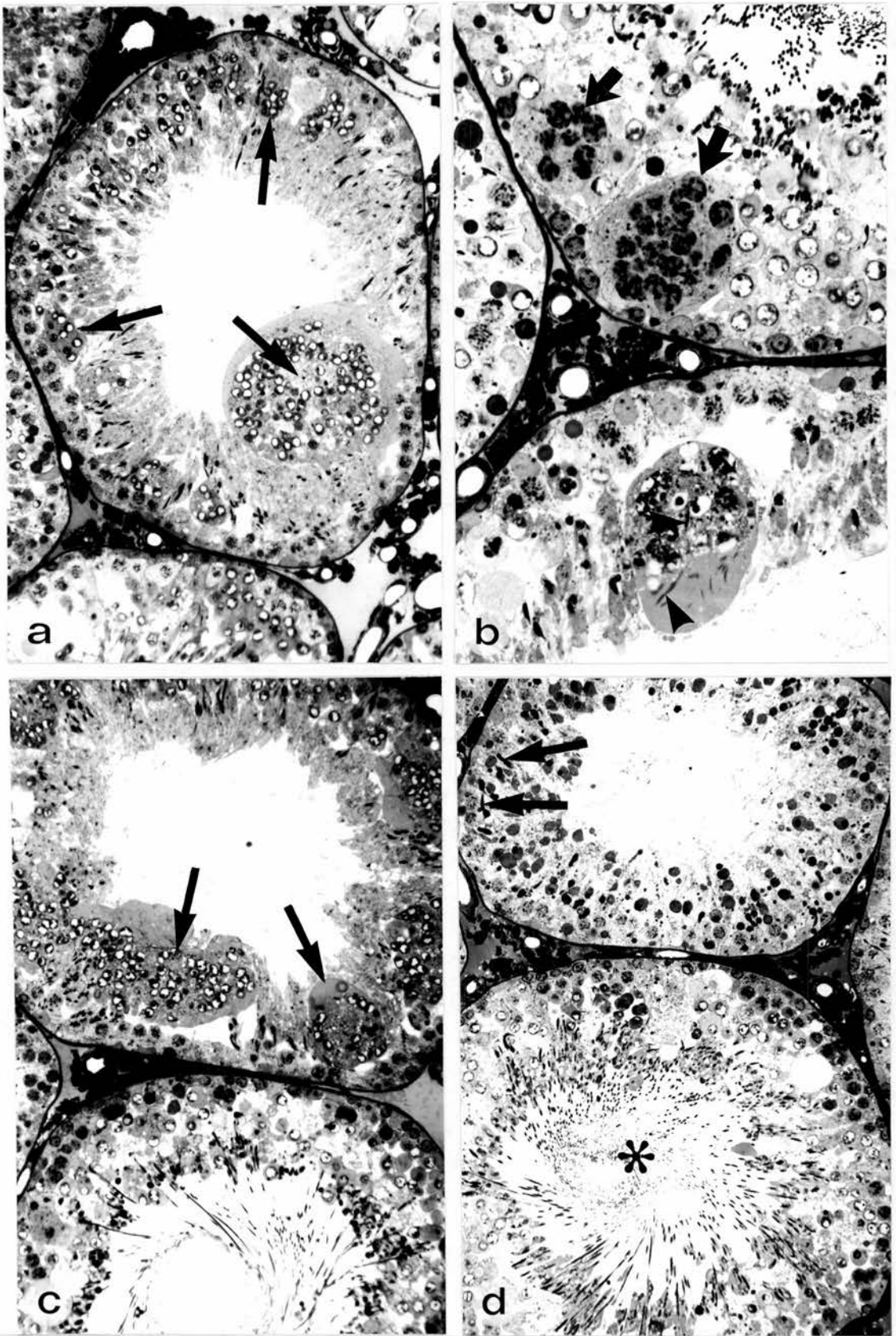
Plate 4.4. Spermatogenesis 72h after a single oral dose of NB showing; (a) giant bodies (arrows) formed from degenerating round spermatids in a stage IV tubule (x190): (b) section through three adjacent seminiferous tubules showing giant bodies formed from pachytene spermatocytes (arrows) and a giant body containing elongate spermatids (arrowheads) (x300): (c) degenerating round spermatids in giant bodies (arrows) (x220); (d) section through two adjacent seminiferous tubules illustrating in one stage X tubule (upper) abnormal elongate spermatids (arrows) while in the other stage VI tubules (lower) elongate spermatids appear normal (astericks) (x220).
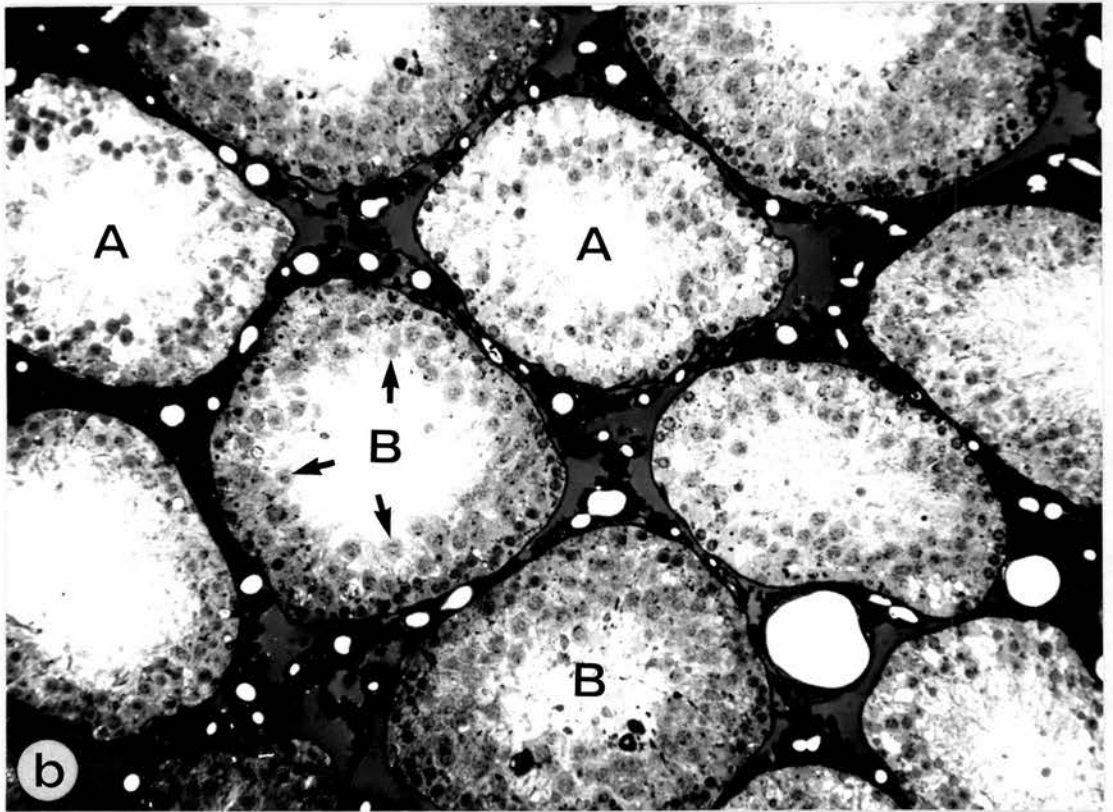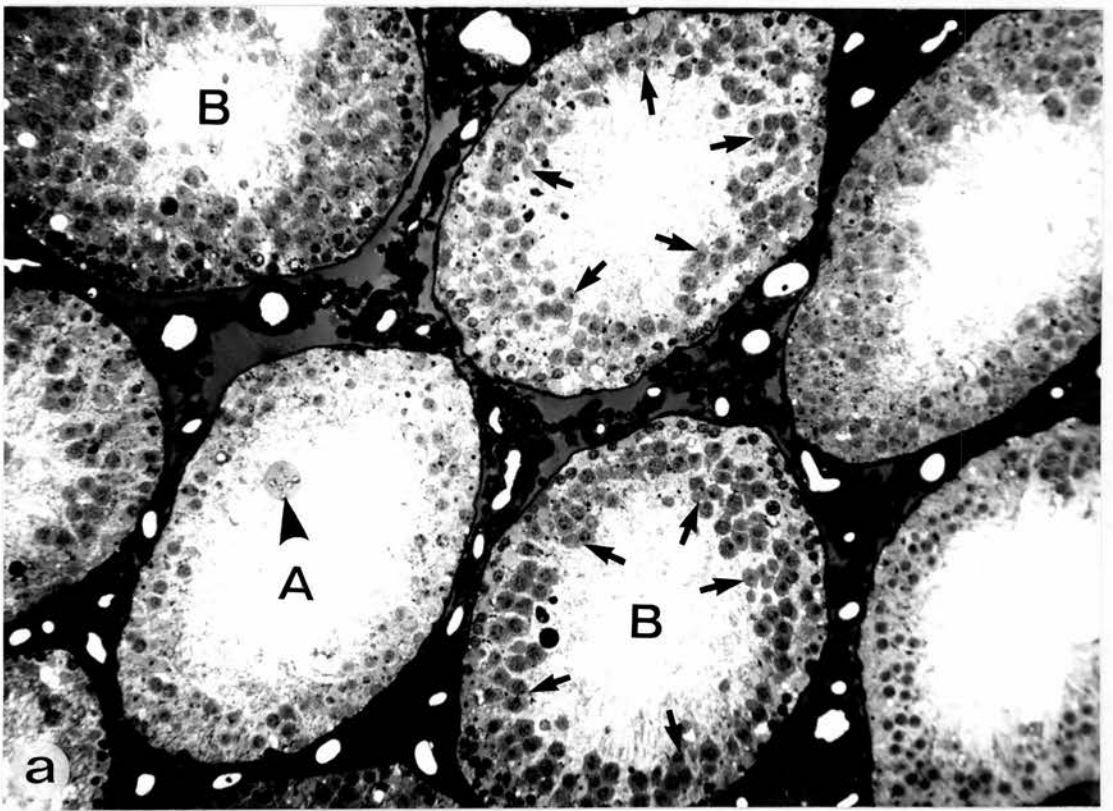
Plate 4.5. Spermatogenesis at 7 days after a single oral dose of NB illustrating (a) seminiferous tubules with differing degrees of regeneration of the epithelium containing pachytene spermatocytes (arrows) whilst giant bodies consisting of elongate spermatids are still present in the lumen of some tubules (arrowheads) (x100): (b) cross section of a number of seminiferous tubules showing different degress of disruption within the epithelium, ranging from severely disrupted (A) to a degree of regeneration (B) (x100).
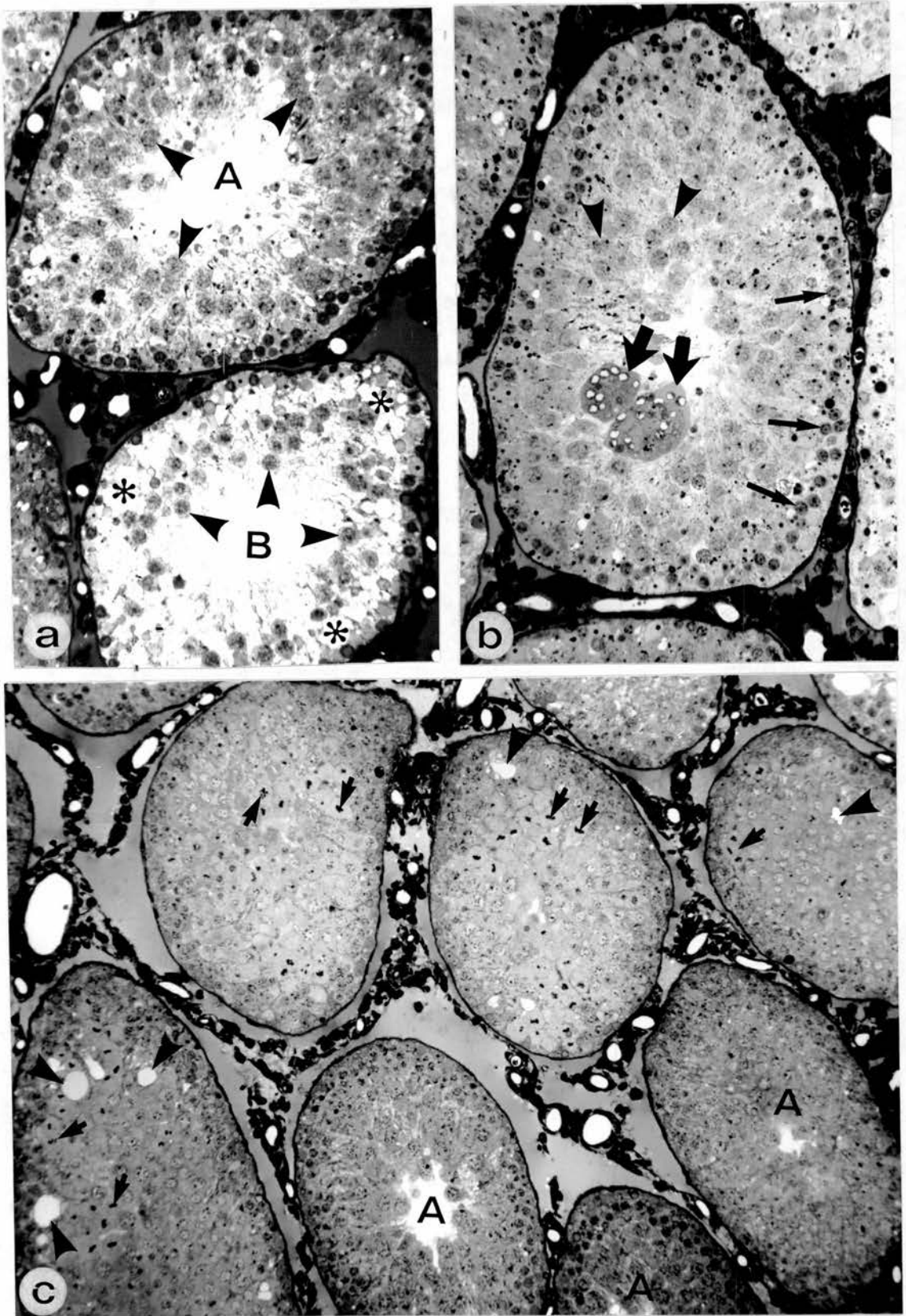
Plate 4.6. Spermatogenesis at 7 days after a single oral dose of NB showing (a) two adjacent seminiferous tubules, the upper with a relatively intact epithelium (A) whilst the lower (B) has a severely disrupted epithelium which is highly vacuolated (astericks); both tubules contain pachytene spermatocytes (arrowheads) (x190): (b) stage XII seminiferous tubule containing numerous pachytene spermatocytes (arrowheads) and leptotene/zygotene spermatocytes (small arrows) but also two giant bodies (large arrows) consisting of degenerating round spermatids (x210): (c) 14 days after a single oral dose of NB illustrating a degree of stage synchrony with a mixture of stage XIII (A) and stage XIV tubules, the latter containing spermatocytes in metaphase (arrows); Note that none of the tubules contain elongate spermatids and vacuoles (arrowheads) are still an abnormal feature of some of the tubules (x100).   142

(plate 4.4. b), in which their appeared to be a giant body which contained the heads of elongate spermatids.

Histological evidence up to 7 days post-treatment therefore suggests that the testicular effects of NB are manifest within 24h, consisting of the degeneration of pachytene spermatocytes in many stages and round spermatids in some stages, while at 3 days post-treatment all germ cells that were pachytene spermatocytes on exposure to NB were degenerating as were all round spermatids irrespective of the stage of the spermatogenic cycle. Due to the kinetics of spermatogenesis it would be impossible for all elongate spermatids to have completed formation and spermiation by 7 days post-treatment, and as they were almost completely absent it would seem that they also degenerated or were released prematurely. These events and the progression of the lesion through spermatogenesis at subsequent time points have been represented schematically in Fig 4.5. The germ cells shown schematically as degenerating at the time of exposure (Fig 4.5.) have been calculated retrospectively from histological observations made at 1, 3 and 7 days post-treatment. However, while all of the germ cell types degenerate or were absent at subsequent time intervals up to 7 days post-treatment, the appearance of a number of germ cells (e.g. elongate spermatids) at earlier time points would suggest that they were morphologically normal at that point.

At 14 days after NB-treatment all tubules contained very few degenerating cells and in most tubules repopulation and expansion of the epithelium was evident (plate 4.6. c). Many tubules contained populations of developing germ cells up to diplotene and secondary spermatocytes in stages XIII and XIV, but round and elongate spermatids were completely absent from all tubules (plate 4.6. c). This was consistent with the progression of the NB-induced lesion through spermatogenesis and the regeneration (between 7 and 14 days post-treatment) of all pachytene spermatocytes (see Fig 4.5). Some tubules also contained extracellular vacuolation (plate 4.8. a) and some degenerating pachytene spermatocytes (not shown), while others appeared histologically normal with the exception of the missing germ cell populations. Cells within the interstitium appeared histologically normal in comparison to controls and it appeared that a possible expansion in the interstitial space or shrinkage of seminiferous tubules had occurred but detailed morphometry is required to evaluate this.

By 21 days after exposure to NB most tubules appeared histologically normal, containing all types of germ cells up to stage VII round spermatids, consistent with the time-dependent regeneration of the epithelium

Fig 4.5. Schematic representation of the spermatogenic cycle in the rat to show the types of germ cells present at each stage and its duration in hours (parentheses). Degenerating and/or absent germ cells observed following histological examination of testes from rats at 24h and at later time points after nitrobenzene administration are represented by the enclosed area within the shaded boxes (⊠). (Time 0 represents control.) In addition, the progression of an increase in the frequency of occurrence of certain stages of the spermatogenic cycle are represented by the speckled areas (▦) shown initially at 3 days after nitrobenzene treatment and at successive time intervals thereafter up to 70 days post-treatment.

Time 0
(CONTROL)

1 Day

3 Days

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
(34.8)	(23.3)	(6)	(13)	(14.7)	(26.5)	(62.8)	(21.3)	(7.1)	(7.1)	(7.1)	(32.3)	(17.6)	(14.1)

Stages of the spermatogenic cycle.
(Duration of each stage (in hours) shown in brackets).

7 Days

14 Days

21Days

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
(34.8)	(23.3)	(6)	(13)	(14.7)	(26.5)	(62.8)	(21.3)	(7.1)	(7.1)	(7.1)	(32.3)	(17.6)	(14.1)

Stages of the spermatogenic cycle.
(Duration of each stage (in hours) shown in brackets).

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
(34.8)	(23.3)	(6)	(13)	(14.7)	(26.5)	(62.8)	(21.3)	(7.1)	(7.1)	(7.1)	(32.3)	(17.6)	(14.1)

Stages of the spermatogenic cycle.
(Duration of each stage (in hours) shown in brackets).

41 Days

70 Days

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
(34.8)	(23.3)	(6)	(13)	(14.7)	(26.5)	(62.8)	(21.3)	(7.1)	(7.1)	(7.1)	(32.3)	(17.6)	(14.1)

Stages of the spermatogenic cycle.
(Duration of each stage (in hours) shown in brackets).

(Fig 4.5.), although elongate spermatids were still absent from all tubules (plate 4.7. a). The round spermatids observed 21 days post-treatment had developed from the diplotene spermatocytes observed at 14 days post-treatment (see Fig 4.5.). Both pachytene spermatocyte and round spermatid populations appeared histologically normal within all tubules possessing these germ cell types (plate 4.7. a), but extracellular vacuolation was still evident in some tubules (plate 4.7. b). In a number of other tubules the process of spermatogenesis had completely broken down. These tubules contained vacuolated Sertoli cells, spermatogonia and only the occasional pachytene spermatocyte (plate 4.7. b).

By 28 days post-treatment most tubules contained an epithelium in which all generations of germ cells were present including elongate spermatids up to step 16 (see plate 4.8. a and Fig 4.5.). The absent step 17 to 19 elongate spermatids represent the remaining lesion created by NB some 28 days earlier. These elongate spermatids developed from the stage I to IV round spermatids observed at 21 days post-treatment (Fig 4.5.). Extracellular vacuolation was observed occasionally in tubules which otherwise appeared histologically normal (plate 4.8. a). However in a minority of tubules spermatogenesis remained completely disrupted (plate 4.8. b and c). The interstitium also appeared histologically normal in comparison to control sections (plates 4.8. d).

At the remaining time points after NB exposure (35, 42 or 70 days) spermatogenesis appeared qualitatively normal in most seminiferous tubules. Within a number of these tubules extracellular vacuolation was observed, although a minority of other tubules either contained only Sertoli cells or had a reduced number or complements of germ cells (plate 4.8. b) when compared to controls (plate 4.8. d).

4.4.7. Evaluation of the effects of NB on the testis using histological observations of epididymal content.

Further indirect identification of testicular dysfunction through the loss of germ cells and an approximation of the duration of dysfunction and severity were determined by histological examination of sections taken from the caput, midpiece and caudal regions of epididymides from NB-treated rats at the same time as testicular tissue. These were compared with equivalent sections of epididymides from rats treated with vehicle alone (plate 4.9. a). These sections revealed no apparent cellular necrosis, and no germ cells of any type other than spermatozoa were present within the caput, midpiece or caudal sections. Histological examination of the epididymides from treated rats revealed no apparent tissue necrosis of the epididymides at any time point however there
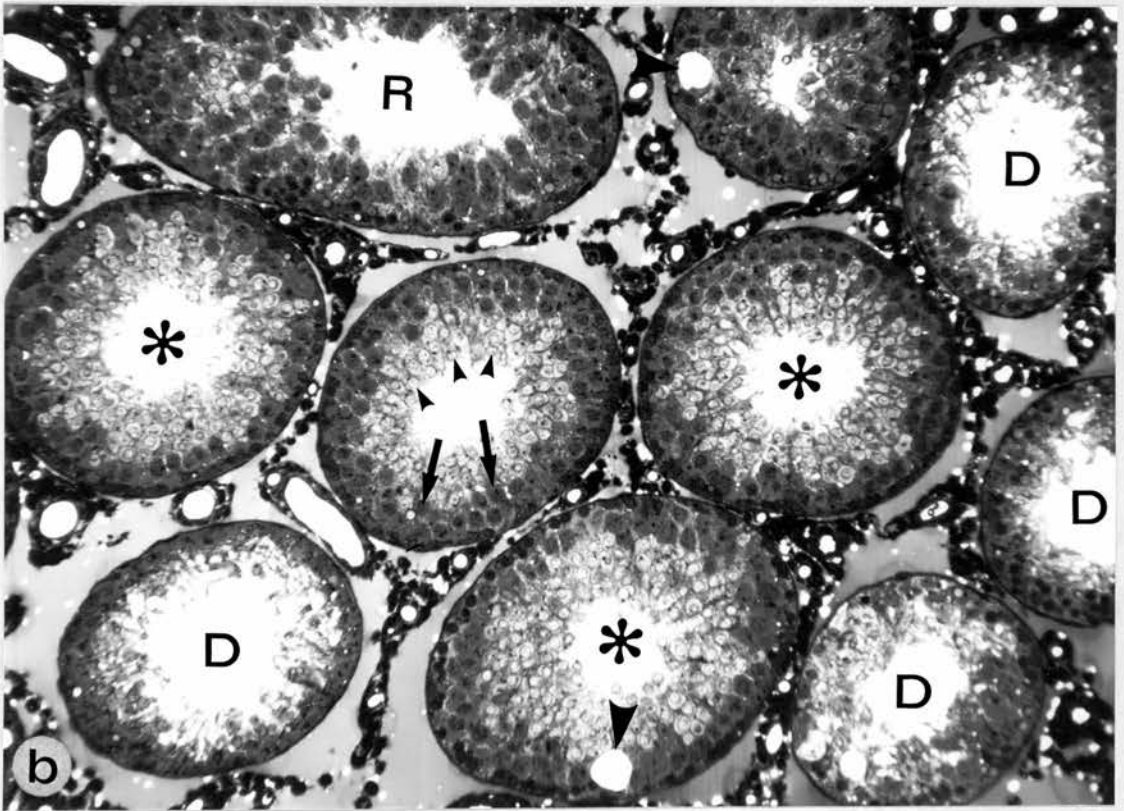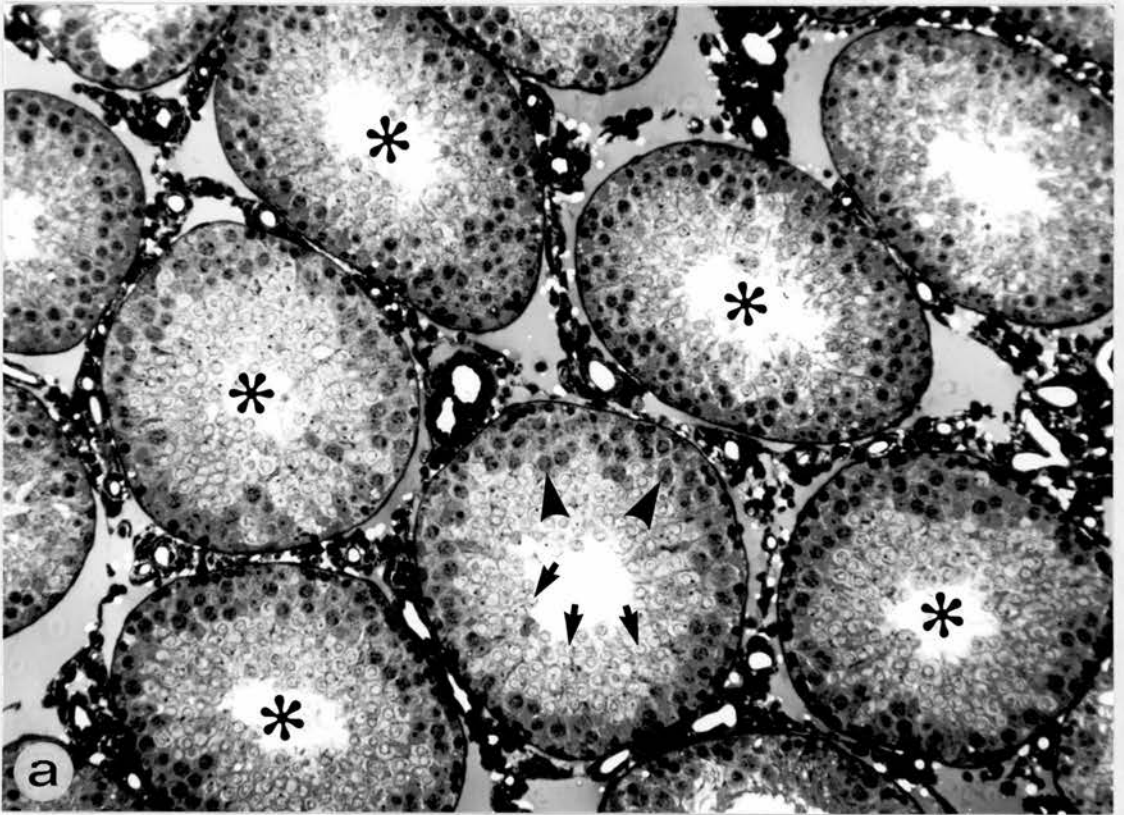
Plate 4.7. Spermatogenesis at 21 days after a single oral dose of NB showing (a) a qualitatively normal epithelium containing pachytene spermatocytes (arrowheads) and round spermatids (arrows) except that there is a complete absense of elongate spermatids (x100): Note also the stage synchrony with all the tubules at stages IV-VIII. (b) seminiferous tubules in a phase of degeneration (D) containing Sertoli cells and some classes of germ cells, or in a phase of regeneration, some tubules containing only pachytene spermatocytes (R) or others containing pachytene spermatocytes (arrows) and round spermatids (small arrowheads); vacuolation within the epithelium was present in some tubules (large arrowheads) (x100).
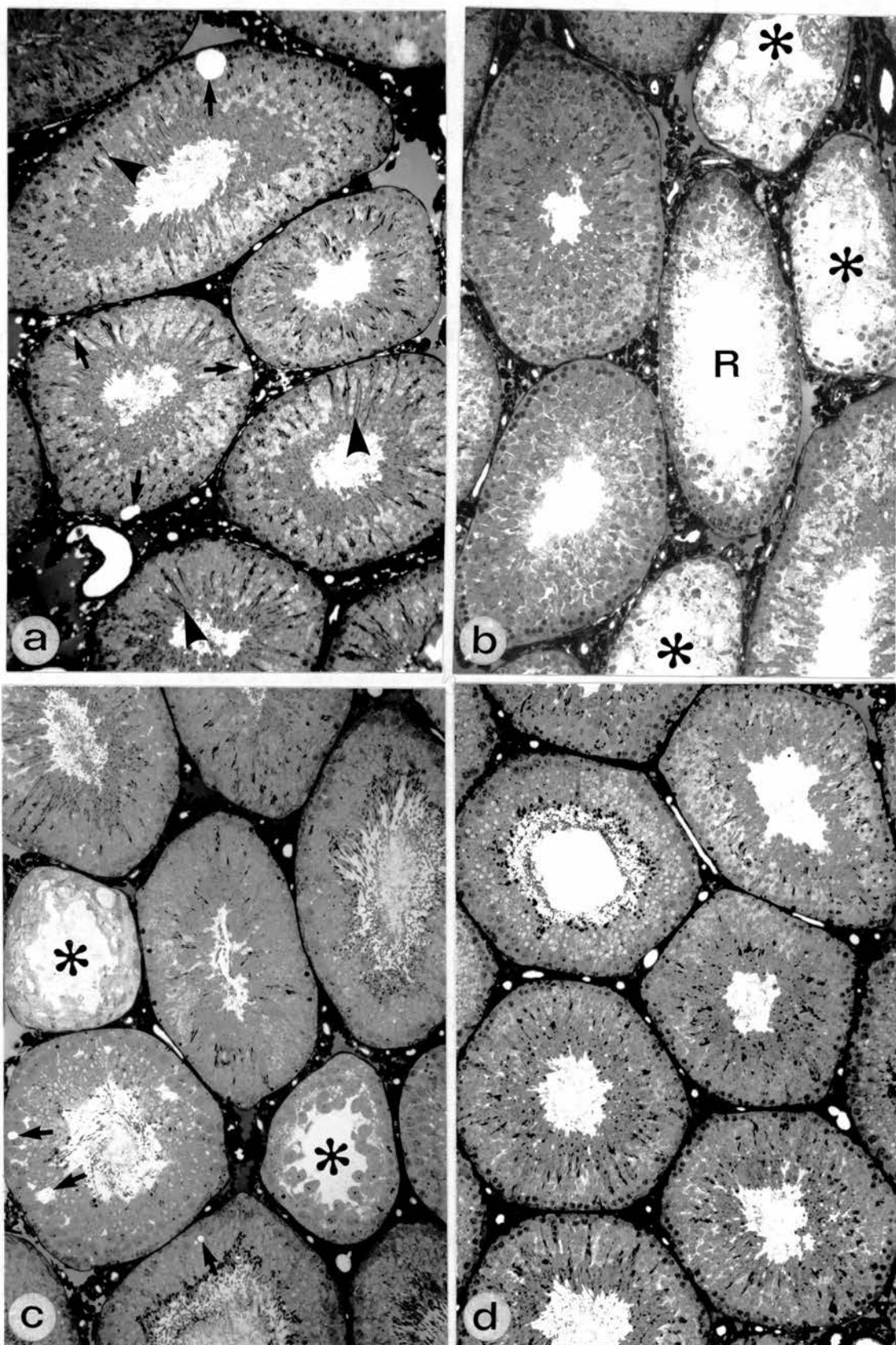
Plate 4.8. Spermatogenesis at 28 days after a single oral dose of NB illustrating (a) qualitatively normal spermatogenesis at stages III-IV with a full complement of germ cells, including elongate spermatids (arrowheads): Note however, that the epithelium of some tubules still contain extracellular vacuoles (arrows); note also the stage synchrony (x 75): (b) in some tubules spermatogenesis is still completely abnormal (astericks) while in others (R) regeneration of the epithelium is only just beginning to occur (x75): (c) spermatogenesis at 70 days after NB treatment illustrating normal spermatogenesis in most tubules with some extracellular vacuolation (arrows) while in other tubules there are only Sertoli cells and few or no germ cells (astericks) (x75): (d) control + 70 days showing completely normal spermatogenesis and an absence of stage synchrony (x75).

were major changes in the content of the epididymides at successive time intervals after dosing.

In the caput region of epididymides removed from rats 24h after NB administration a number of types of germ cells were observed together with numerous sperm (plate 4.9. b). These cells could not be identified with certainty but appeared similar to pachytene spermatocytes; a number of these cells were degenerating. Within the midpiece and caudal regions of the epididymis only sperm were observed.

Within 3 days of NB exposure sperm and other types of germ cells were identified in small numbers in the caput section of the epididymides taken from these animals (plate 4.9. c). These cells were similar in appearance to pachytene spermatocytes and round spermatids which represented the largest populations of germ cells sloughed from the epithelium of the seminiferous tubules at this time (see plates 4.2. a and c). The midpiece sections of epididymides taken from animals 3 days after NB treatment contained sperm and occasionally degenerating round spermatids or possibly pachytene spermatocytes. However the caudal sections of epididymides taken from these animals were histologically normal in comparison to controls, containing only sperm.

The caput sections of epididymides taken from rats 7 days after NB treatment contained a number of types of degenerating round cells (probably different types of germ cells) and giant bodies (plate 4.9. d). A decrease in the number of sperm in the caput section of epididymides taken from treated rats also appeared to have occurred in comparison to controls. Within the midpiece and the caudal epididymal sections degenerating germ cells (mainly pachytene spermatocytes and round spermatids) were also present; their numbers were greater in the caput and midpiece than in the caudal regions in sections taken from the same epididymis.

Within 14 days of NB administration the caput section of epididymides taken from NB-treated rats contained numerous degenerating germ cells and giant bodies (plate 4.10. a). The midpiece and caudal sections of these epididymides also contained a number of germ cells other than sperm, although their numbers were greater in the midpiece than in the cauda. The cauda also contained numerous elongate spermatids.

At 21 days after NB exposure the caput region of the epididymides of treated rats were devoid of most degenerating germ cells and all sperm (plate 4.10. b). The midpiece region of these epididymides contained numerous degenerating germ cells and very few sperm, while the caudal region
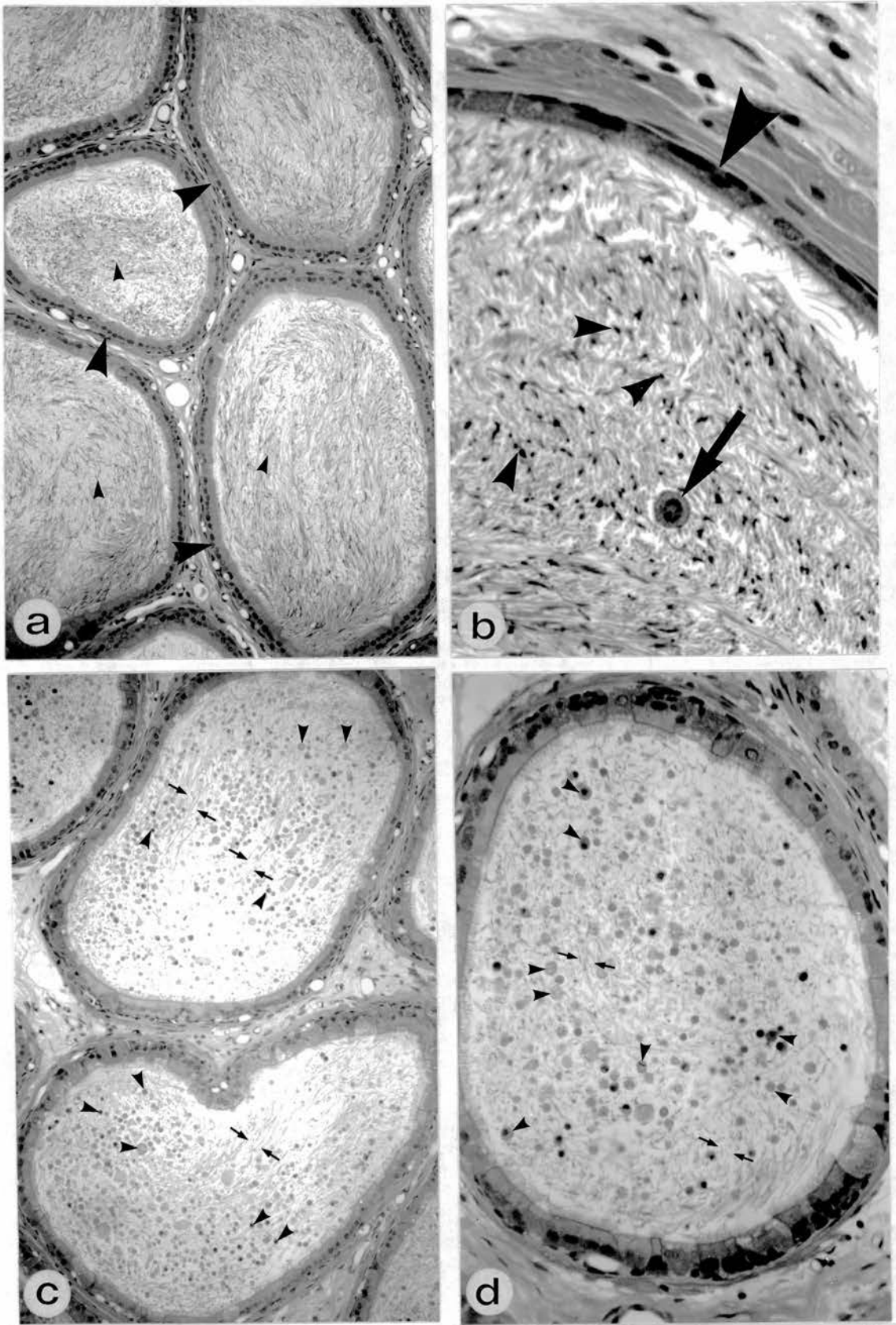
Plate 4.9. Section through epididymides (a) from a control animal showing epididymal tubules (large arrowheads) containing numerous sperm within the lumen (x75): (b) 1 day after a single oral dose of NB illustrating the epithelium of the caput region of epididymal tubule (large arrowhead) and an immature germ cell (round spermatid?) within the lumen (arrow) amongst sperm (small arrowheads) (x270): (c) 3 days after NB administration showing numerous degenerating germ cells (arrowheads) and sperm (arrows) within the caput epididymis (x75): (d) 7 days post NB-treatment illustrating numerous degenerating germ cells (arrowheads) amongst sperm (arrows) in the cauda epididymal region (x190).

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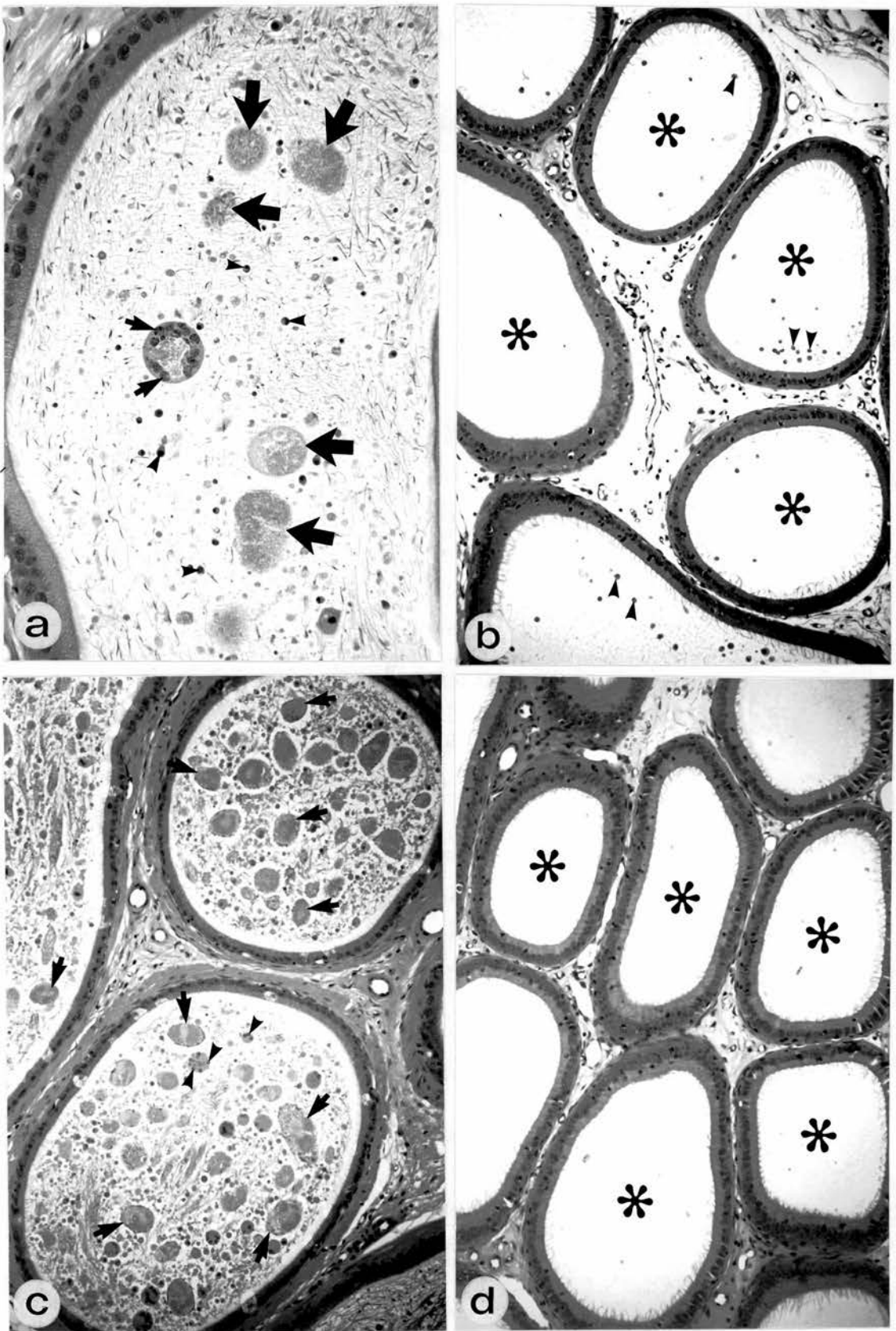
Plate 4.10. (a) Caput epididymal lumen 14 days after a single oral dose of NB illustrating numerous degenerating giant bodies (large arrows) containing identifiable germ cells (small arrows) amongst individual degenerating germ cells (arrowheads) and sperm (x190): (b) caput region of the epididymis at 21 days after NB-treatment showing an almost empty lumen (astericks) except for occasional degenerating germ cells (arrowheads) (x75): (c) midpiece region of the epididymis at 28 days after NB-treatment illustrating numerous giant bodies (arrows) containing identifiable germ cells (arrowheads) (x75): (d) caput region of the epididymis at 28 days after NB-treatment showing a complete absence of germ cells and sperm (x75).

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contained more degenerating germ cells, giant bodies (in comparison to 14 days post-treatment) and numerous sperm (plate 4.10. c).

Within 28 days of NB treatment the caput region remained devoid of germ cells and all sperm (plate 4.10. d) whilst the midpiece and caudal region of these epididymides contained numerous degenerating germ cells and numerous sperm (not shown).

At 35 days after NB treatment a small number of sperm were observed in the caput region of the epididymides removed from a number of rats. The midpiece region of these epididymides were devoid of sperm and other germ cells, while the caudal sections contained numerous degenerating germ cells in addition to sperm and were similar in appearance to caudal sections observed in NB treated rats at 28 days post-treatment. Even at 41 days after NB treatment when sperm were reappearing in the caput and midpiece regions (not shown), the caudal region contained numerous degenerating germ cells and numerous sperm (not shown).

Comparative observations made on the density of germ cells and sperm in each section of epididymides taken from treated and control rats were purely qualitative. Further quantitative work would be required to substantiate the apparent changes in the density of sperm at successive time intervals after treatment. However, these histological observations do substantiate those made in connection with testicular histological changes with respect to the degree of damage caused by NB, and its duration.

4.4.8. Effect of NB on the kinetics of spermatogenesis

During the initial 7 days post-treatment the damage created by NB to all stages of the spermatogenic cycle resulted in a shift in the frequency of occurrence of stages such that, at 7 days post-treatment (and possibly also at 3 days), numerous tubules were classified histologically into one or two successive defined stages of the spermatogenic cycle. At subsequent time intervals these changes in the frequency of occurrence of specific stages were still apparent (Fig 4.6): For example, at 14 days post-treatment a significant increase in the number of stage XIII to XIV tubules was observed in comparison to the frequency of occurrence of stage XIII and XIV in testicular cross sections in control animals (see Fig 4.6 and plates 4.6. b, 4.7. a and 4.8. a). In addition, at 41 days post-treatment a significant increase in the number of stage III tubules was particularly evident under histological examination (not shown).

Due to the fixed duration of each stage of the spermatogenic cycle it was then possible to calculate and predict which stages had increased or

a) **3 Days**

(r=0.75)

b) **7 Days**

(r=0.62)

I - IV   V - VI   VII - VIII   IX - XI   XII - XIV

Groups of spermatogenic stages
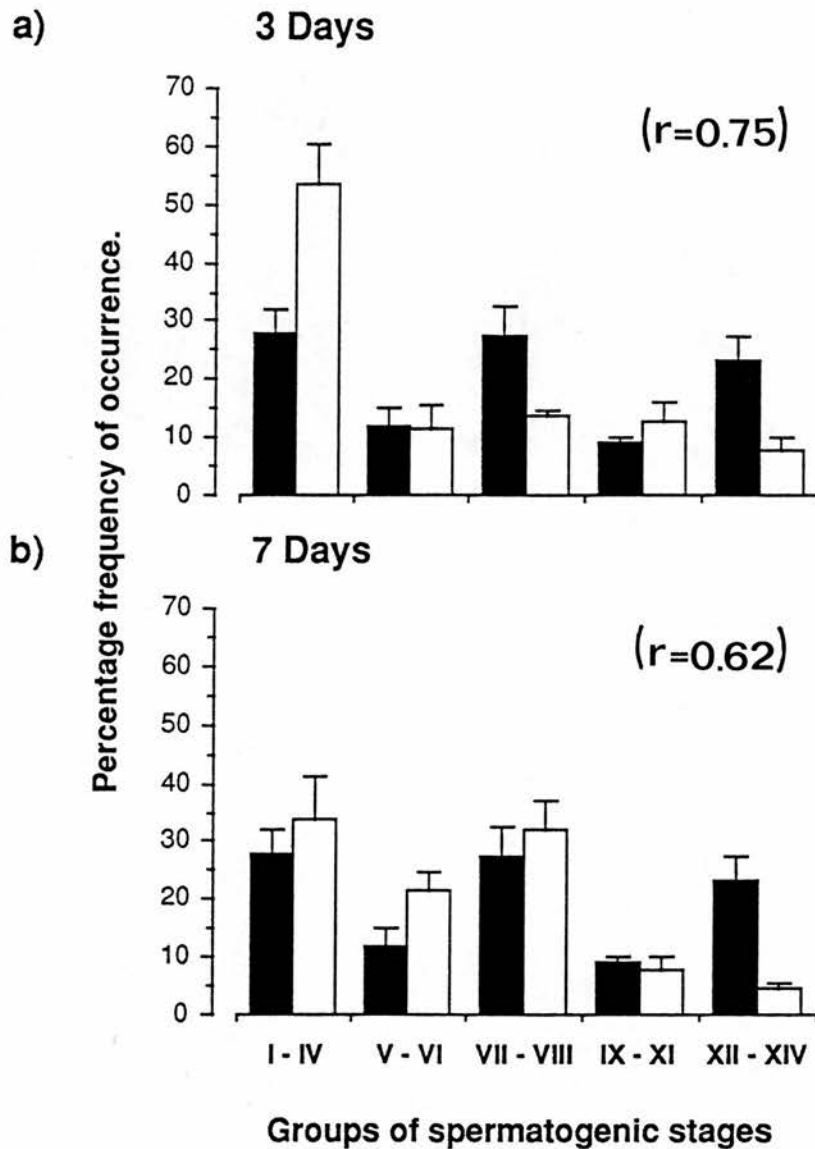
Fig 4.6. Effect of a single oral dose of nitrobenzene (300 mg/kg bodyweight) on the precentage frequency of occurrence of specific groups of stages in sections from testes of treated rats (□) versus those counted in vehicle treated controls (■): (Since the frequency of occurrence of stages in control rats at each time point did not vary significantly, data was pooled and a representative frequency for each group of stages in controls shown on each graph). Values are means ± SD for 100 seminiferous tubule cross sections staged/animal with 2 animals/time point for NB- or vehicle-treated rats at 3 days (**a**), 7 days (**b**), 14 days (**c**), 21 days (**d**), 28 days (**e**), 35 days (**f**), 42 days (**g**) and 70 days (**h**). Two-way Analysis of Variance with replication was performed on data and significances are shown in parenthesis.

c) **14 Days** (r=0.81)

d) **21 Days** (r=0.78)

e) **28 Days** (r=0.84)

Percentage frequency of occurrence.

I - IV   V - VI   VII - VIII   IX - XI   XII - XIV

Groups of spermatogenic stages

f)

35 Days

(r=0.74)

g)

41 Days

(r=0.92)

h)

70 Days

(r=0.67)

I - IV   V - VI   VII - VIII   IX - XI   XII - XIV

Groups of spermatogenic stages

Percentage frequency of occurrence.
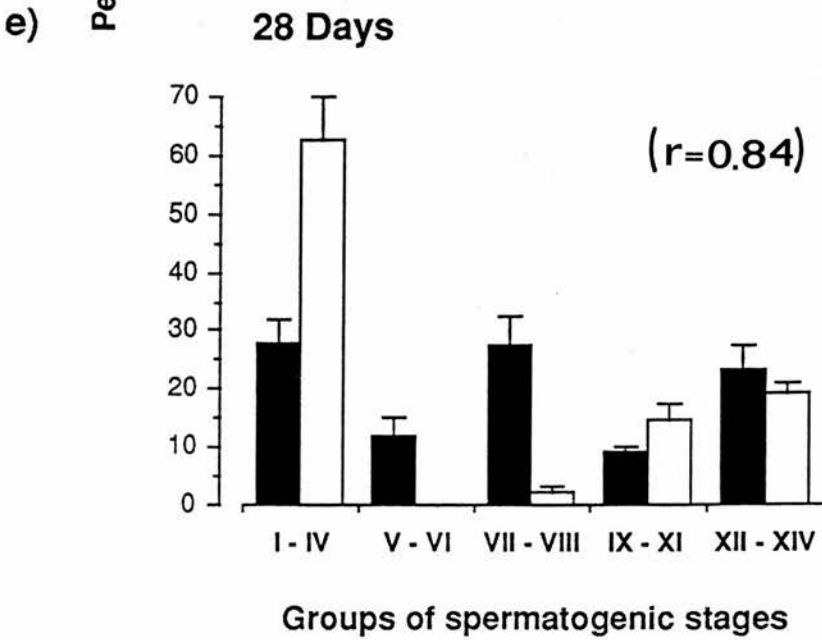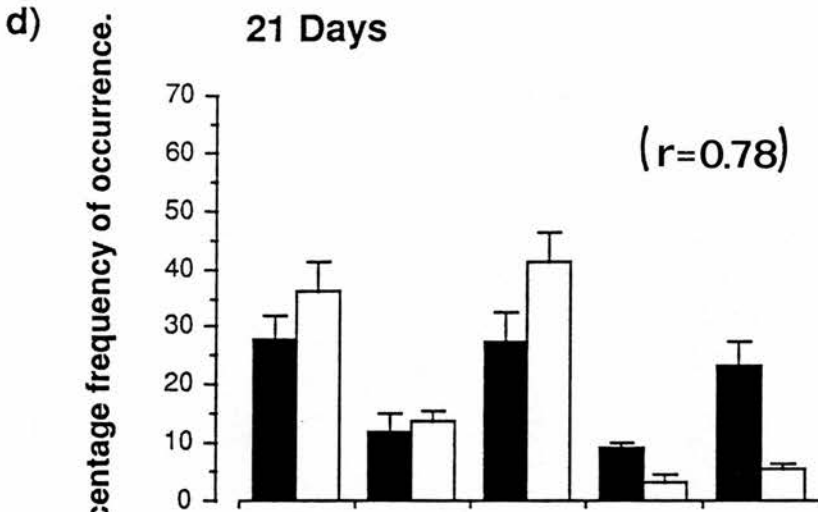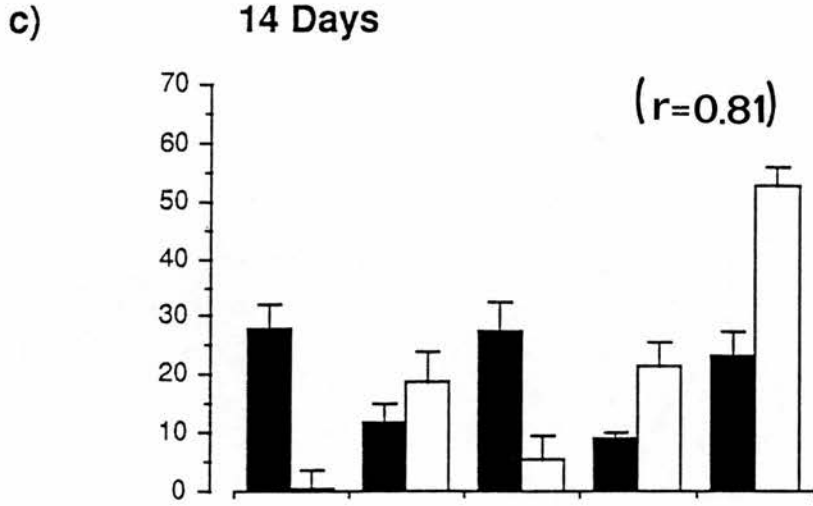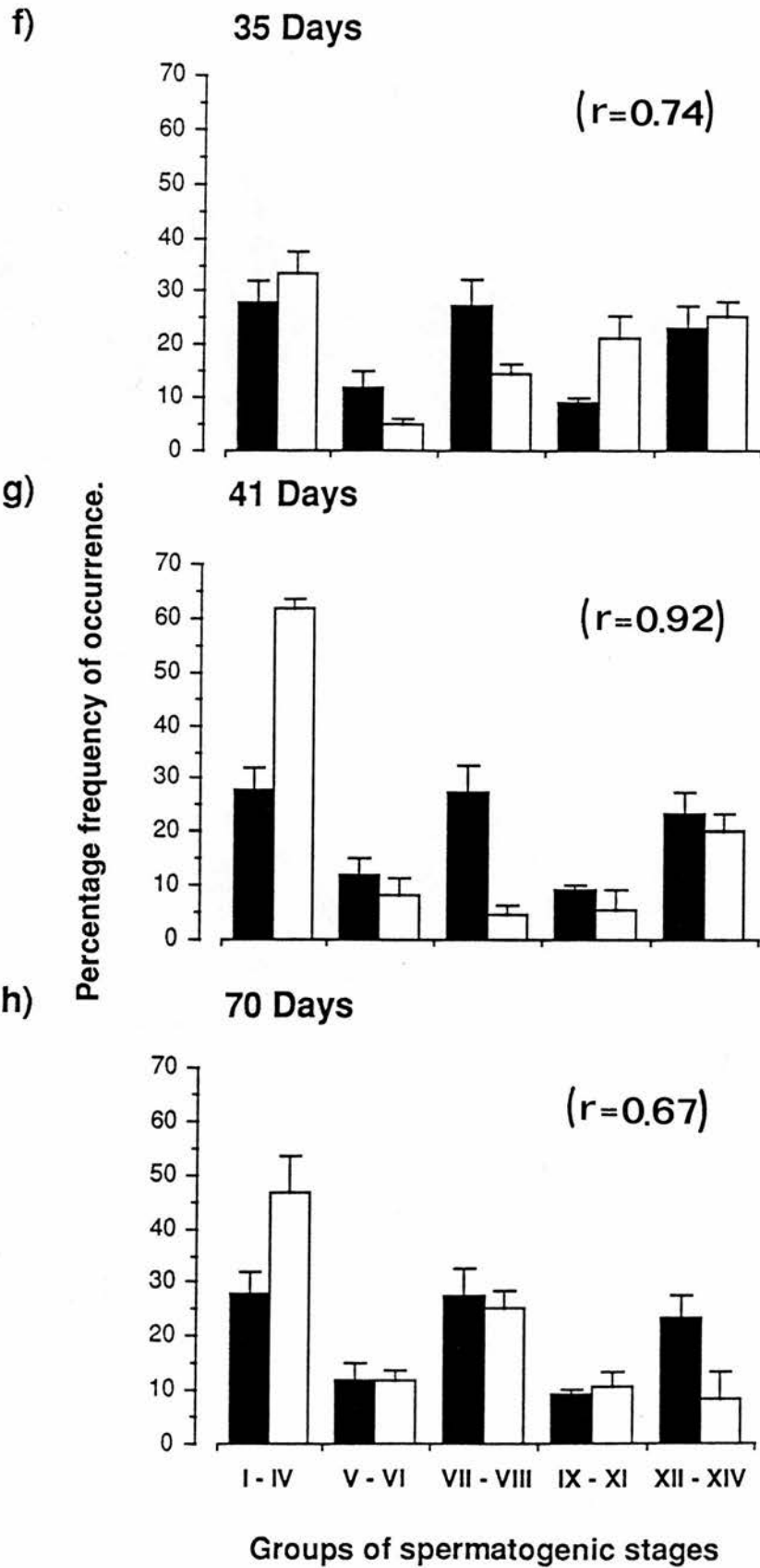
decreased their frequency based on the results obtained at earlier time points. Verification of these predictions were determined by histological examination to stage and count the frequency of occurrence of particular stages at successive time intervals after NB administration (see Fig 4.6). Based on the results obtained in Fig 4.6 a schematic representation of the spermatogenic cycle in the rat together with the duration of each stage at successive intervals after dosing are shown in Fig 4.5. The progression of the most significant synchrony of stages is shown as the stippled area in each illustration.

Based on the significant increase in the frequency of occurrence of stages I to IV and the significant decrease in the frequency of stages XIII and XIV observed at 3 days post-treatment (Fig 4.6), an interruption of the spermatogenic wave at stage IV would appear to have occurred in NB treated rats such that many tubules developed to, but did not progress through, stages I to IV at the normal rate.

By 7 days post-treatment the increase in the frequency of occurrence of stages I to IV (particularly stages I and II), was manifest as a significant increase in stages V and VI and a smaller rise in stages VII and VIII, while the frequency of stages XIII and XIV was significantly reduced (Fig 4.6). Predictions based on the frequency of occurrence of stages at 3 days post-treatment reinforce the changes observed following histological examination of cross sections taken 7 days after NB administration (see Fig 4.5.).

At 14 days post-treatment a significant increase in the frequency of occurrence of stages IX to XI and stages XIII and XIV were observed (Fig 4.6). The latter group of stages was particularly apparent under histological examination (see plates 4.6. b, 4.7. a and 4.8. a). The total duration of these two stages is 31.7h while the spermatogenic cycle lasts 287.7h (Fig 4.5.). This increased frequency of stages XIII and XIV was derived from the earlier increases in stages V and VI, and stages VII and VIII at 7 days post-treatment (Fig 4.6), and were predicted from the progression of the increase in frequency of stages I to IV 96h earlier (Fig 4.5.). Significant decreases were also observed in the frequency of occurrence of stages VII and VIII at 14 days post-treatment while stages I to IV were almost totally absent.

By 21 days post-treatment, significant increases in the frequency of stages VII and VIII, and decreases in stages XIII and XIV, and stages IX to XI were observed in comparison to the frequency of occurrence measured in control rats (Fig 4.6). By 28 days post-treatment a significant increase in stages I to IV was evident, while stages V and VI were totally absent and stages VII and VIII were greatly reduced in number. Between 35 and 70 days post-

treatment most tubules contained a full complement of germ cells, however a small percentage were classified as Sertoli cell only, containing Sertoli cells and a number of spermatogonia. The change in the frequency of occurrence of certain stages of the spermatogenic cycle was still evident at 70 days post-treatment (Fig 4.6). These changes in the pattern of occurrence of stages at these and other time intervals after NB treatment were predictable from changes in frequency at earlier time points i.e. from 72h after treatment (see Fig 4.5.) This assumes that the normal kinetics of spermatogenesis were affected by NB only up to 72h post-exposure. Since it was possible to accurately predict stage synchrony in advance of morphological examination it is probable that the kinetics of spermatogenesis were unaffected by NB after this 72h period.

4.4.9. Effect of NB on Sertoli cell morphology and germ cell exfoliation *in vitro*

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Control cultures of Sertoli cells and Sertoli cell-germ cells were considered to be normal under histological examination 24h after the administration of DMSO to the cultures (plate 4.11. a). Confluent monolayers of Sertoli cells were formed in culture within 3 days of plating. Co-cultures appeared morphologically normal with no apparent germ cell necrosis or significant cellular exfoliation of germ cells or Sertoli cells induced by the vehicle (plate 4.11. a). Sertoli cell monocultures also appeared confluent following DMSO administration with no observable vacuolation within Sertoli cells or germ cells.

Following exposure of Sertoli cell monocultures or Sertoli cell-germ cell co-cultures to NB for 24h, dose-dependent morphological changes were observed in culture (plate 4.11. b, c and d). In co-cultures, Sertoli cell vacuolation coupled with an observable decrease in the density or absence of different populations of germ cells was evident from visual examination of the co-cultures exposed to NB at doses up to 10^{-3}M (plate 4.11. b, c and d) when compared with vehicle-treated control cultures (plate 4.11. a). NB caused a dose-dependent decrease in the density of germ cells in co-culture. Morphological examination of co-cultures exposed to doses in excess of 5×10^{-5}M NB exhibited Sertoli cell phagocytosis of a number of degenerating germ cells of different classes (plate 4.11. b). Dose-dependent vacuolation of Sertoli cells was also observed in monocultures of Sertoli cells with doses of NB identical to those which resulted in Sertoli cell vacuolation in co-culture. In both co-culture and monoculture the administration of 5×10^{-3}M NB resulted in gross morphological changes (not shown). These included the breakdown of
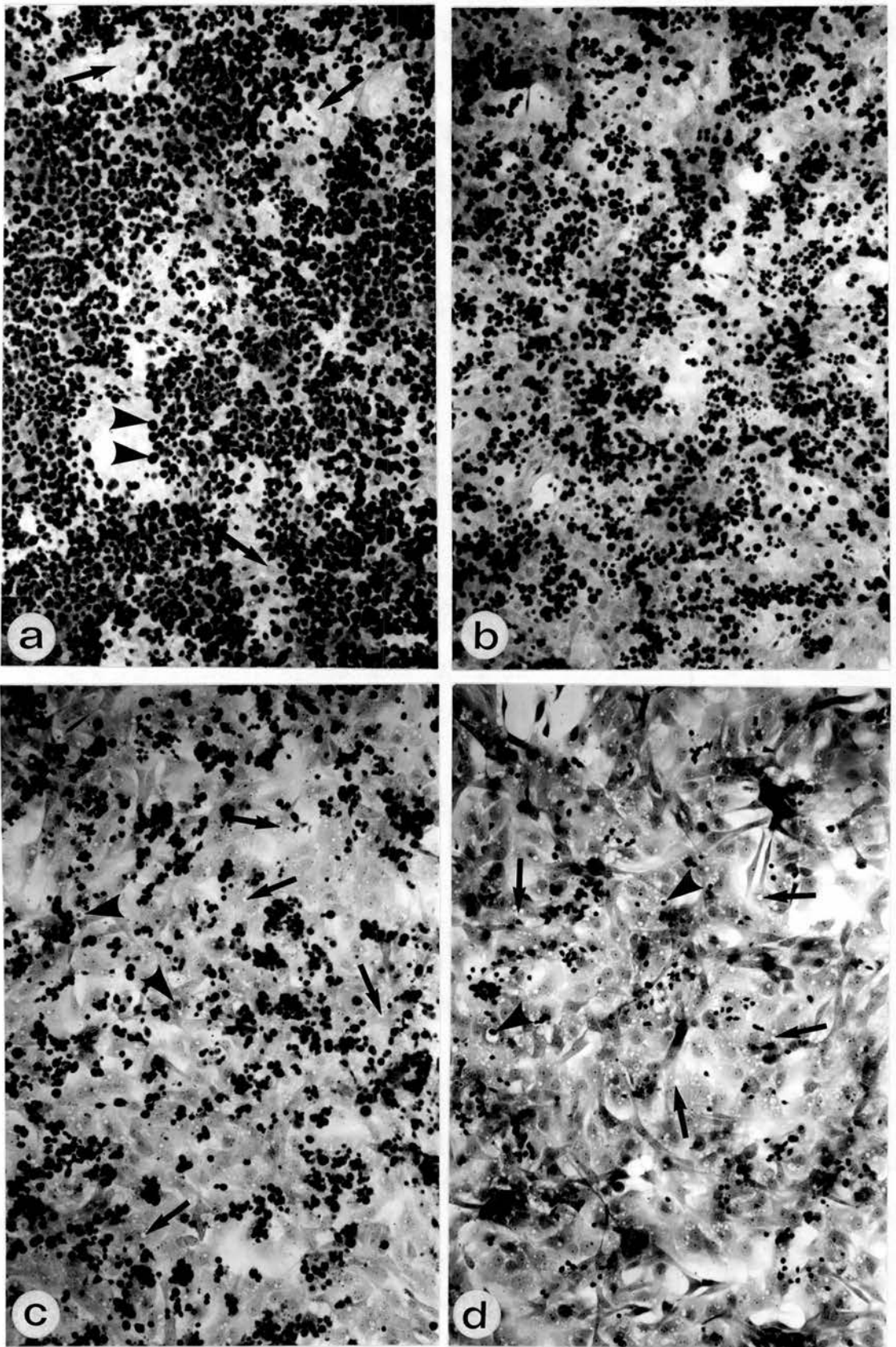
Plate 4.11. Sertoli-germ cell co-cultures following exposure for 24h to (a) control vehicle (DMSO-0.3% v/v), showing confluent Sertoli cells (arrows) beneath numerous adherent clusters of germ cells (arrowheads); (b) 5×10^{-5}M NB, showing a marked decrease in the density of adherent germ cells together with phagocytosis of degenerating germ cells by Sertoli cells (arrowheads) and vacuolation of the confluent monolayer of Sertoli cells (arrows); (c) 5×10^{-4}M NB, illustrating Sertoli cell vacuolation (arrows) and more pronounced loss of adherent germ cells; (d) 10^{-3}M NB, showing extensive germ cell loss, phagocytosis of degenerating germ cells (arrowheads) and vacuolation (arrows) of the confluent Sertoli cell monolayer. Haematoxylin and Papanicolaou's (x75).

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the confluent monolayer of Sertoli cells resulting in the appearance of large holes in the culture and coupled with significant vacuolation within both Sertoli cells and germ cells of different classes.

Exposure of co-cultures and monocultures to *meta* dinitrobenzene (mDNB) at the single dose level used (10^{-4}M) also induced a marked decrease in the density of germ cells in co-culture in comparison to vehicle treated control co-cultures, coupled with Sertoli cell vacuolation in both co-cultures and monocultures. While only a single toxic dose was used for comparison as a positive control in this study, the effects of mDNB on Sertoli cell monocultures and co-cultures were discussed in greater detail in Chapter 3.

The effect of NB administration on germ cell exfoliation from Sertoli cell-germ cell co-cultures is shown in Fig 4.7. Following exposure to NB for 24 h, a dose-dependent exfoliation of cells was observed (Fig 4.7), which was significantly ($P<0.001$) greater than the exfoliation of cells from control co-cultures at doses in excess of 5×10^{-6}M (5 μM) NB. These exfoliated cells were considered to be primarily germ cells since treatment of co-cultures with 5×10^{-4}M (500 μM) NB resulted in significant exfoliation of cells ($380 \times 10^3 \pm 8.8 \times 10^3$M total cells/well) whereas an insignificant number of exfoliated cells ($1.4 \times 10^3 \pm 0.4 \times 10^3$ total cells/well) was measured after the exposure of Sertoli cell monocultures to an identical dose of NB. In the latter instance, the exfoliated cells represented only 0.4% of the cells in the culture well and were presumed to represent the detachment of residual germ cells that contaminated Sertoli cell monocultures following Tris treatment. Exposure of co-cultures to mDNB at the single dose level used (10^{-4}M), resulted in greater exfoliation of germ cells when compared to that measured after treatment with an equimolar dose of NB (Fig 4.7). Control co-cultures treated with vehicle alone (DMSO) showed a significant ($P<0.01$) increase in detachment of cells when compared to non-treated co-cultures.

In DMSO-treated cultures, $96 \pm 1\%$ (mean $\pm$ SD) of the exfoliated cells excluded trypan blue, indicating that they were essentially viable (see Table 4.1.). After exposure to low doses of NB (up to 10^{-4}M or 100μM on Fig 4.8) the viability of exfoliated cells was similarly high (87 to 91%) but it decreased marginally with higher dose of NB [$83 \pm 4\%$ at 5×10^{-4}M (500μM) and $80 \pm 5\%$ at 10^{-3}M (1000μM)] and after exposure to 10^{-4}M (100 μM) mDNB ($84 \pm 4\%$). After exposure of co-cultures to 5×10^{-3}M (5000μM) NB the percentage of exfoliated cells excluding trypan blue was decreased substantially ($65 \pm 6\%$ - see Table 4.1). This dose was therefore considered cytolethal for germ cells.

Fig 4.7. Effect of a range of doses of nitrobenzene (■), *meta* -dinitrobenzene (mDNB; 10⁻⁴M) (□) or control vehicle (DMSO-0.3% v/v) (▨) on the number of detached cells present in the medium of Sertoli cell-germ cell co-cultures after a 24h exposure period. Values are means ± SD for 6 replicates (*P<0.001, in comparison to control). Results of a representative experiment are shown.

Table 4.1. The effect of a range of doses of NB (0.01 - 5000μM), a single dose of mDNB (100μM or 10^{-4}M) or control vehicle (DMSO - 0.3% v/v) on the viability of exfoliated cells removed from the medium of co-cultures 24h after compound administration. Cell counts were performed using a haemocytometer and non-viable cells were identified using 0.05% trypan blue solution; cells which excluded trypan blue were considered viable. Values are means ± SD for six replicate cultures (*P<0.001, compared to control). Results of a representative experiment are shown.

Compound and dose (μM)		Percentage of exfoliated cells absorbing trypan blue dye (mean ± SD)
DMSO	(0.3%v/v)	4.0 ± 1.3
mDNB	100	16.2 ± 3.7*
NB	0.01	6.6 ± 1.7
NB	0.05	7.8 ± 1.1
NB	0.1	8.8 ± 2.5
NB	0.5	9.2 ± 3.6
NB	1.0	9.6 ± 2.7
NB	5.0	10.9 ± 4.0
NB	10	11.3 ± 2.8*
NB	50	12.0 ± 3.4*
NB	100	13.1 ± 4.1*
NB	500	16.6 ± 4.3*
NB	1000	19.7 ± 4.9*
NB	5000	35.2 ± 5.7*

4.4.10 Effect of NB on the secretion of lactate and pyruvate by Sertoli cells in culture.

The addition of NB at doses in excess of 5×10^{-4}M in co-culture or 10^{-3}M in monoculture or mDNB in both culture systems resulted in significant dose-dependent increases in the secretion of lactate and pyruvate (Fig 4.8.). In the presence of germ cells the increase in both lactate and pyruvate secretion induced by NB occurred at slightly lower doses (5×10^{-4}M), while at higher doses the magnitude of lactate and pyruvate secretion was consistently greater in the absence of germ cells (Fig 4.8.). Exposure of cultures to mDNB also increased lactate and pyruvate secretion and again responses were consistently enhanced in the absence of germ cells (Figs 4.8.). In both co-culture and monoculture the effect of 5×10^{-3}M NB (considered a germ cell

Fig 4.8. Effect of a range of doses of nitrobenzene (■), *meta* -dinitrobenzene (mDNB; 10^{-4}M) (□) or control vehicle (DMSO-0.3% v/v) (▩) on the secretion of lactate (**a**) and pyruvate (**b**) by Sertoli cell-germ cell co-cultures (left column) or Sertoli cell cultures (right column) over a 24h exposure period. Values are means ± SD for 6 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a representative experiment are shown.
Note however that the Sertoli cell cultures and co-cultures were derived from separate cell preparations. Labels for intermediate doses of nitrobenzene on the abscissa (eg 5×10^{-8} to 5×10^{-3}M) have been omitted for clarity.

cytolethal dose as judged by trypan blue exclusion) was approximately equivalent in magnitude to that obtained after exposure to 10^{-4}M mDNB (considered a Sertoli cell toxic but non-cytolethal dose).

4.4.11. Effect of NB on inhibin secretion by Sertoli cells in culture.

The effect of NB on Sertoli cell inhibin secretion in co-culture and monoculture is shown in Fig 4.9. After exposure to NB a biphasic response of basal inhibin secretion was observed in both Sertoli cell monocultures and Sertoli cell-germ cell co-cultures. Low doses of NB (5×10^{-8} to 10^{-6}M) stimulated inhibin secretion, intermediate doses (5×10^{-5} to 5×10^{-4} M) had no effect, whereas high doses (10^{-4} to 10^{-3}M) stimulated inhibin secretion (Fig 4.9.). This biphasic response of Sertoli cells to NB was observed in 4 out of 5 separate culture preparations (data not shown). Although the biphasic pattern of inhibin secretion induced by NB was, in general, independent of the absence or presence of germ cells, the magnitude of inhibin secretion in both toxicant treated (at most doses) and control cultures was consistently and significantly greater ($P<0.01$ to $P<0.001$) in Sertoli cell-germ cell co-cultures than cultures of Sertoli cells only (see Figs 4.9., 4.12. and 4.13.). The exceptions to this occurred in co-culture (Fig 4.9.) at 5×10^{-4} and 1×10^{-3}M NB when basal inhibin secretion was significantly elevated ($P<0.01$ and $P<0.001$ respectively) in comparison to both control secretion and monocultures at identical doses of NB. Although the magnitude of inhibin secretion by cultures varied between experiments, increased secretion of inhibin in the presence of germ cells was observed in a number of independent experiments utilising separate cell preparations and a number of cultures derived from the same initial cell preparation cultured in the presence of NB (Fig 4.9.), in the presence of increasing concentrations of FSH (Fig 4.12.) or in the presence of FSH and NB (Fig 4.13.). At the single dose level used, mDNB had no effect on the basal secretion of inhibin by Sertoli cells in either the presence or absence of germ cells (Fig 4.9.).

4.4.12. Effect of FSH on lactate and pyruvate secretion by Sertoli cells in culture.

Small but significant ($P<0.01$) dose-dependent increases in the secretion of lactate and pyruvate were evident following FSH administration (Fig 4.10.). The FSH-stimulated levels of lactate and pyruvate were always greater in monoculture than co-culture (see Fig 4.10.).
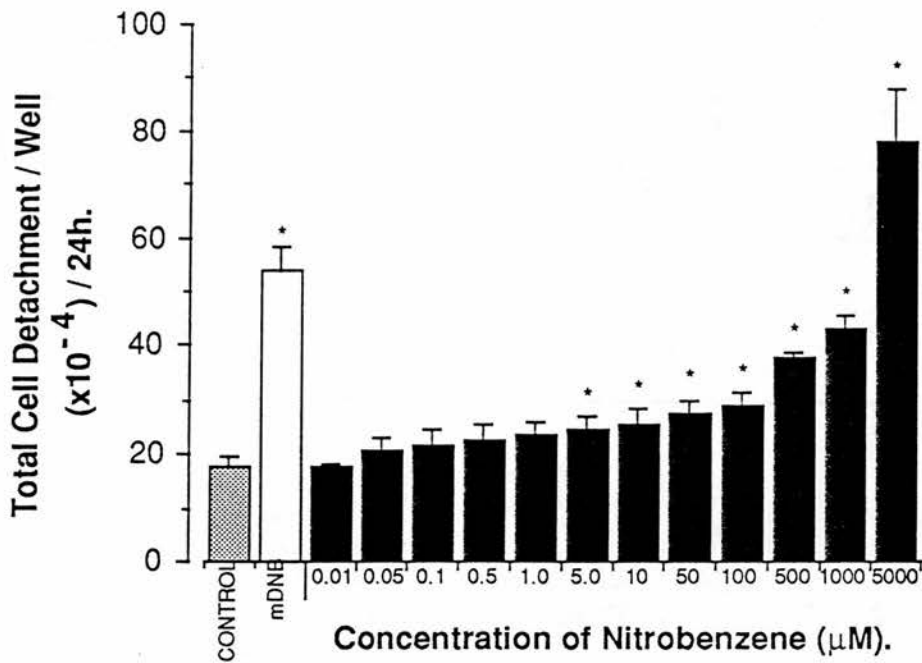
Fig 4.9. Effect of a range of doses of nitrobenzene (■), *meta* -dinitrobenzene (mDNB; 10^{-4}M) (□) or control vehicle (DMSO-0.3% v/v) (▨) on the secretion of $^{1-26}\alpha$-inhibin by Sertoli cell-germ cell co-cultures (a) or Sertoli cell cultures (b) over a 24h exposure period. Values are means ± SD for 6 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a representative experiment are shown. Note however that the Sertoli cell cultures and co-cultures were derived from separate cell preparations. Labels for intermediate doses of nitrobenzene on the abscissa (eg 5×10^{-8} to 5×10^{-3}M) have been omitted for clarity.

Fig 4.10. Effect of a range of doses of porcine follicle stimulating hormone (FSH) (■), on the secretion of lactate (a) and pyruvate (b) by Sertoli cell-germ cell co-cultures (left column) or Sertoli cell cultures (right column), or basal secretion (□) over a 24h exposure period. Values are means ± SD for 6 replicates (*P<0.01, in comparison to respective controls). Results of a representative experiment are shown. Note that both Sertoli cell cultures and co-cultures were derived from the same initial cell preparation.

A dose-dependent increase in lactate and pyruvate secretion was evident in both monoculture and co-culture, reaching their highest levels at an FSH concentration of 1.7×10^{-2} U/well (Fig 4.10.). The administration of higher concentrations of FSH did not increase the secretion of lactate or pyruvate from both monoculture or co-culture.

4.4.13. Effect of NB on FSH-stimulated secretion of lactate and pyruvate by Sertoli cells in culture.

FSH is one of the primary physiological modulators of Sertoli cell function (including the secretion of lactate, pyruvate and inhibin) and, *in vivo*, Sertoli cells are constantly exposed to FSH. It was therefore assessed whether the toxic effects of a number of doses of NB and the single dose of mDNB on basal Sertoli cell function described earlier for lactate, pyruvate and inhibin secretion were affected by concomitant stimulation with FSH.

In the presence of FSH, the addition of NB at high doses (10^{-3}M and the germ cell cytolethal dose of 5×10^{-3}M) to either Sertoli cell monocultures or co-cultures resulted in significant (P<0.001), dose-dependent increases in the levels of lactate and pyruvate in the medium (Fig 4.11.). The levels of lactate and pyruvate secreted by both control and NB-treated co-cultures and Sertoli cell monocultures were increased to a small but significant extent (P<0.05) by FSH in comparison to non-stimulated cultures (for lactate and pyruvate compare Figs 4.8. and 4.10. respectively). However the pattern and magnitude of response to NB of both lactate and pyruvate (Fig 4.8.) secretion remained unchanged in the presence of FSH.

mDNB also induced significant (P<0.001) increases in the levels of lactate and pyruvate (Fig 4.8.) in the medium secreted from both co-culture and monoculture, and these were significantly greater in magnitude than NB-induced increases. Again, the pattern of lactate and pyruvate secretion in FSH-stimulated cultures was comparable to that in non-stimulated cultures following mDNB administration.

4.4.14. Effect of FSH stimulation on inhibin secretion by Sertoli cells in monoculture and co-culture.

Dose-dependent increases in inhibin secretion by both co-culture and Sertoli cell monocultures were observed following FSH administration in comparison to non-stimulated respective controls (Fig 4.12.).

These increase were significant (P<0.01) at 1.7×10^{-3} and 1.7×10^{-2} U FSH/well for monocultures and co-cultures respectively. Again a significant
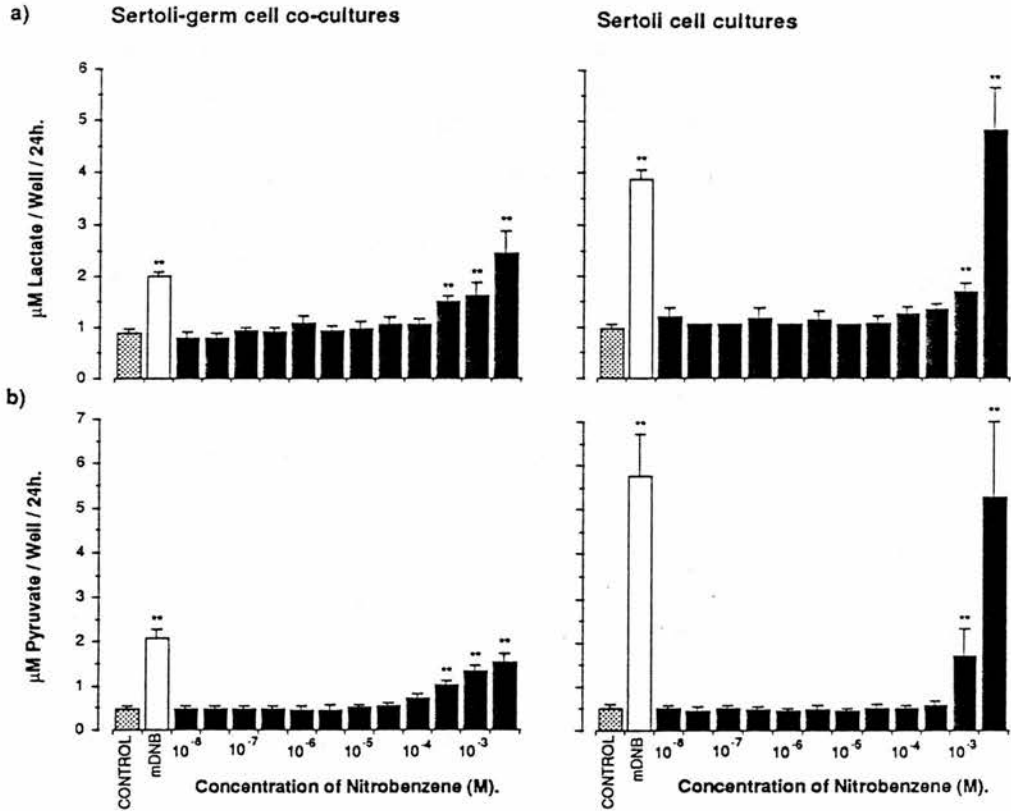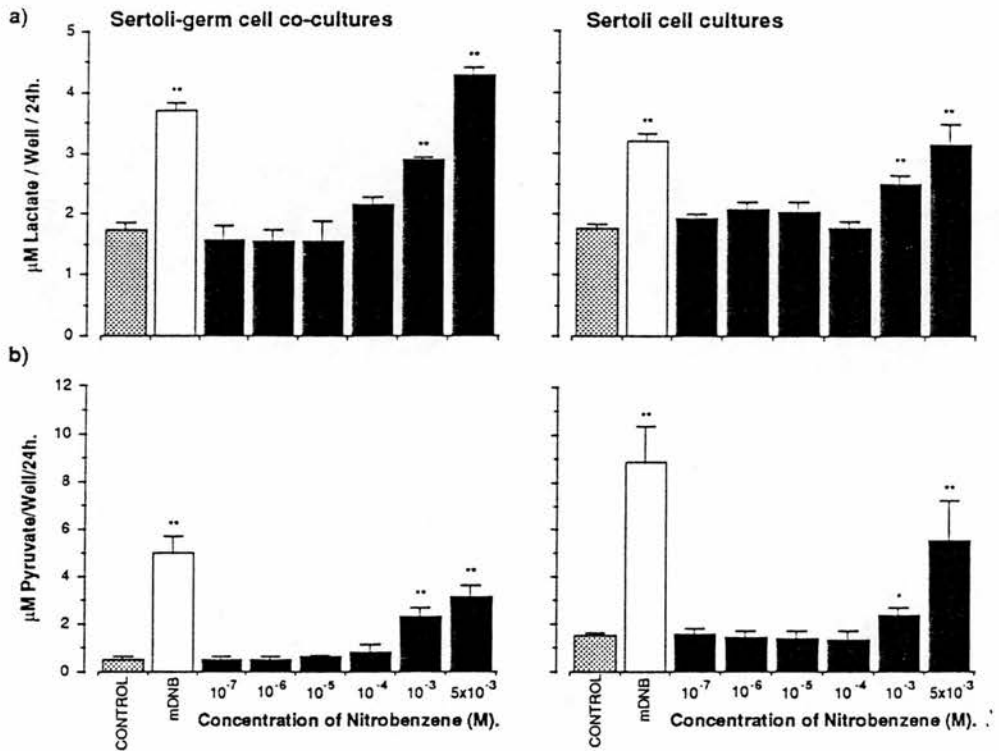
Fig 4.11. Effect of a range of doses of nitrobenzene (□), *meta* -
dinitrobenzene (mDNB; 10^{-4}M) (■) or control vehicle (DMSO-0.3% v/v) (▨)
on the secretion of lactate (a) and pyruvate (b) by Sertoli cell-germ cell
co-cultures (left column) or Sertoli cell cultures (right column) over a 24h
exposure period in the presence of 0.021U porcine follicle stimulating hormone
(FSH)/well. Values are means ± SD for 6 replicates (*P<0.01,**P<0.001, in
comparison to respective controls). Results of a representative experiment are
shown. Note however that the Sertoli cell cultures and co-cultures were derived
from separate cell preparations.
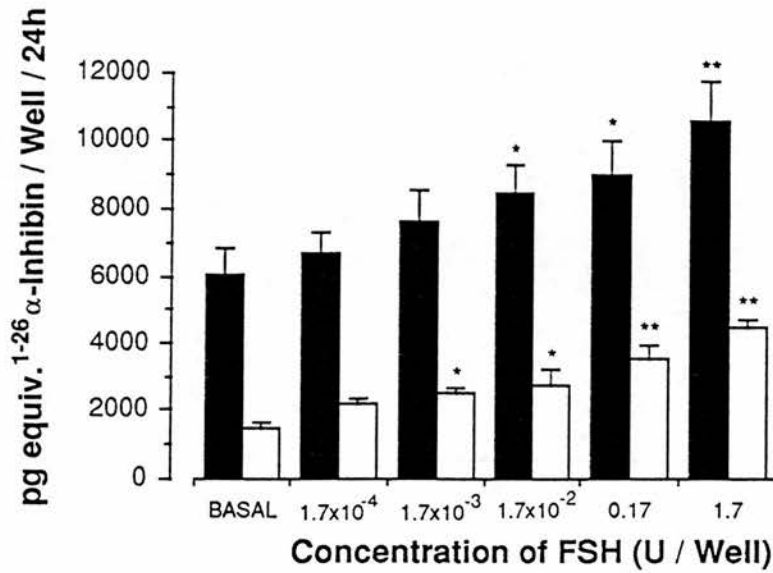
Fig 4.12. Effect of a range of doses of porcine follicle stimulating hormone ⋮ (FSH) on $^{1-26}\alpha$-inhibin secretion in Sertoli cell-germ cell co-cultures (■) or Sertoli cell cultures (▨). Values are means ± SD for 6 replicates (*P<0.01, **P<0.001, in comparison to respective basal values). Results of a representative experiment are shown. Note that both Sertoli cell cultures and co-cultures were derived from the same initial cell preparation.

difference (P<0.001) was measured between the levels of inhibin stimulated by FSH in co-culture and Sertoli cell monoculture. These two- to three- fold differences between co-culture and monoculture were consistent between experiments (see Fig 4.12.). Therefore germ cells enhanced both basal and FSH-stimulated Sertoli cell inhibin secretion in culture.

4.4.15. Effect of NB on FSH-stimulated inhibin secretion by Sertoli cells in culture.

In contrast to the biphasic effect of NB on inhibin secretion under basal conditions (Fig 4.9.) no such pattern was evident in FSH-stimulated co-cultures or Sertoli cell monocultures (Fig 4.13.) and the only significant effect of NB was a marked attenuation (P<0.01) of the FSH-induced increase in inhibin secretion when added at 5×10^{-3}M.

A similar pattern of response was observed following the administration of 10^{-4}M mDNB to both co-cultures and monocultures in the presence of FSH (Fig 4.13.). mDNB, at the single toxic dose level used, negated the FSH-stimulated inhibin response (P<0.001) to a similar degree as did 5×10^{-3}M NB (Fig 4.13.).

4.5. DISCUSSION.

The results from the present study are the first to specifically identify in detail the initial target and subsequent events which occur within the testis of rats following a single oral dose of NB. It is also the first study to measure and correlate hormonal alterations with histological changes at serial time points after administration of NB. Furthermore, by studying the effects of NB on Sertoli cells in monoculture and co-culture the likely target cell for NB action has been identified using a number of known indices of Sertoli cell dysfunction in culture including changes in morphology, germ cell exfoliation and increases in the secretion of lactate and pyruvate. In addition inhibin, a Sertoli cell glycoprotein secreted in response to FSH and thought to regulate FSH secretion *in vivo* and *in vitro*, has been assessed following NB administration as a further index of Sertoli cell function both *in vitro* and *in vivo*.

Early investigations of testicular toxicity of NB were performed on Fischer 344 rats by Bond *et al* (1981). Single oral administration of NB (300-450 mg/kg) produced testicular lesions including germ cell necrosis, multinucleated giant cells and a decrease in sperm number in the epididymis. Subsequent studies have established the reversible nature of NB-induced
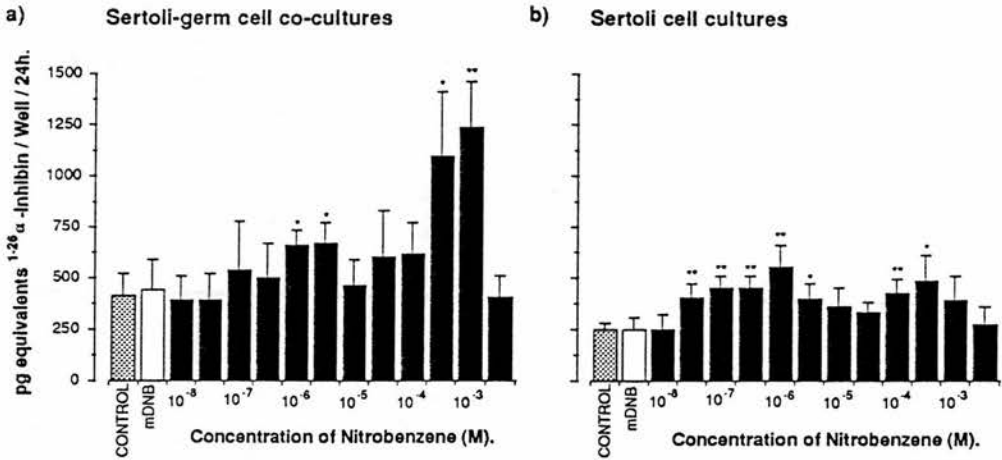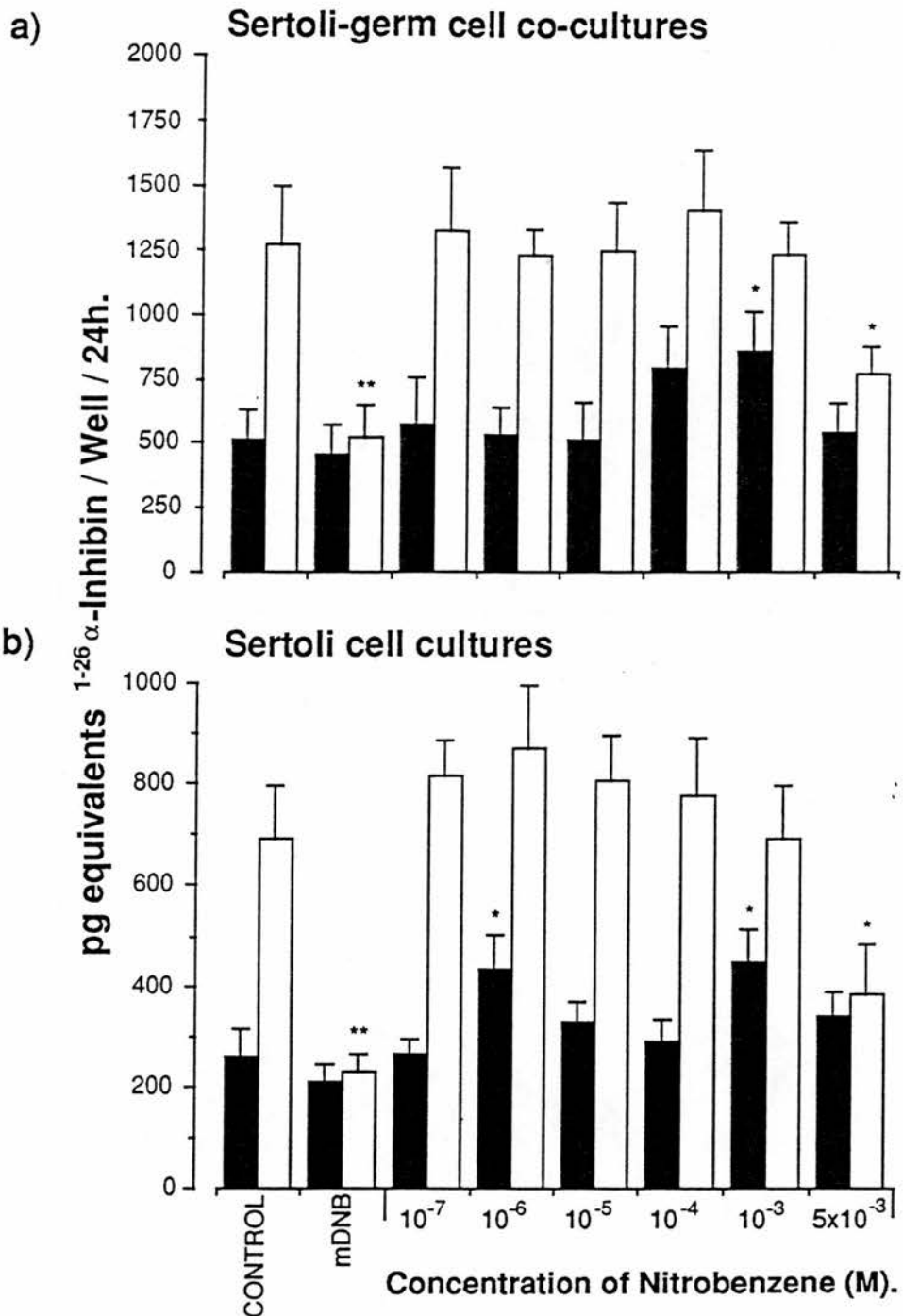
Fig 4.13. Effect of a range of doses of nitrobenzene, *meta* -dinitrobenzene (mDNB; 10^{-4}M) or control vehicle (DMSO-0.3% v/v) on the secretion of $^{1-26}\alpha$-inhibin by Sertoli cell-germ cell co-cultures (a) or Sertoli cell cultures (b) over a 24h exposure period in the absence (■) or presence (□) of 0.021U porcine follicle stimulating hormone (FSH)/well. Values are means ± SD for 6 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a representative experiment are shown. Note however that the Sertoli cell cultures and co-cultures were derived from separate cell preparations.

testicular degeneration produced via oral or inhalation administration in a number of strains of rats but not mice (Medinsky & Irons, 1982; Levin *et al*, 1983; Hamm *et al*, 1984; Dodd *et al*, 1987; Levin *et al*, 1988). However, to date it has not been established whether the deleterious effects of NB on spermatogenesis after *in vivo* administration are the result of an effect(s) on the testis directly or are secondary to an effect elsewhere in the body (eg. the pituitary). If the initial site and mechanism of action of NB-induced spermatogenic disruption are directly on the testicular cells, then further investigations of this toxicity *in vitro* are required. No studies on the potential toxicity of NB *in vitro* on testicular cells have been published.

The effects of NB *in vivo*, identified at a histological level in this study, were manifest within 24h of administration and consisted of Sertoli cell vacuolation coupled with the loss and/or degeneration of pachytene spermatocytes. These two initial events were consistent with other published data on the morphological events observed 24h after treatment with NB (Bond *et al*, 1981). The testicular degeneration induced by the closely related dinitrobenzenes (particularly mDNB) is considered to result from toxic effects on Sertoli cells (see Chapter 3 and Foster *et al*, 1986; 1987; Blackburn *et al*, 1988). Pachytene spermatocyte degeneration was observed initially at 24h post-treatment and was stage dependent, being confined to stages VII to XII.

Concomitant with these histological changes 24h after NB administration serum FSH levels were raised and testicular IF levels of inhibin were also elevated in comparison to controls. Raised serum FSH levels are widely considered to reflect major disruption of normal testicular function and germ cell degeneration *in vivo* (de Krester & Kerr, 1983). Historically, FSH has been postulated to positively regulate Sertoli cell inhibin production. Recent evidence suggests that in the adult male, inhibin levels usually increase and decrease in parallel with changes in FSH (Lincoln *et al*, 1989). Following NB administration increases in both serum FSH and IF inhibin were observed 24h after treatment, the latter changes almost certainly occurring in response to the former as judged by several other studies on the hormonal control of IF levels of inhibin (Sharpe *et al*, 1988; Sharpe & Maddocks, 1989; Sharpe & Maddocks, 1990). Alternatively, it is possible that inhibin secretion into IF may have been increased as a direct result of NB action on the Sertoli cell although the studies *in vitro* do not provide clear cut evidence for such an effect.

Based on histological examination of testes from animals 72h after NB administration, a number of other events had occurred. In addtion to Sertoli cell vacuolation, extracellular vacuolation of the epithelium had occurred. These

vacuoles or spaces probably represented pachytene spermatocytes and other germ cells which were either sloughed into the lumen, or more likely, phagocytosed by Sertoli cells. Furthermore pachytene spermatocytes in stages I to IV which appeared normal 24h post-treatment were now almost absent or degenerating. By comparison to control tissue, round spermatids in spermatogenic stages I to IV appeared abnormal 72h after NB treatment. The nucleus of the round spermatids appeared fragmented. In a number of tubules at 1 day post-treatment giant bodies consisting of coalesced pachytene spermatocytes were observed and at 3 days post-treatment giant bodies made up of either pachytene spermatocytes or round spermatids were observed. The nuclei of round spermatids within these structures contained fragmented chromatin. Within many of these giant bodies it was also possible to identify elongate spermatids but impossible to evaluate if these were normal in appearance. The composition of giant bodies was confined to round spermatids or pachytene spermatocytes. In no section were round spermatids and pachytene spermatocytes observed to coalesce into the same body.

The formation of these giant bodies of degenerating germ cells are a common phenomenon in testicular dysfunction induced by several toxicants. These include vitamin A deficiency (Mitranond *et al*, 1979), or exposure to 2-5 hexanedione (Chapin *et al*, 1982), methylchloride (Chapin *et al*, 1984) or mDNB (Hess *et al*, 1988). These giant bodies probably represent the coalescence of germ cell clones which were linked by intracellular bridges (Dym & Fawcett, 1971; Chapin *et al*, 1983) prior to NB treatment. This conclusion is reinforced by the fact that only giant bodies consisting of pachytene spermatocytes or round spermatids could be identified, since intracellular bridges only exist between germ cells of the same type at any one stage of the spermatogenic cycle.

By 7 days post-treatment a significant compression of the epithelium in NB-treated rats had occurred as a result of the magnitude of germ cells missing from the testis and this was reflected in a significant reduction in testicular weight. A decline in testicular weight is characteristic of testicular toxicants which significantly reduce germ cell number.

Histological examination of testes from animals 7 days post-treatment showed pachytene spermatocytes (in stages VII to XII) and all round spermatids were completely absent. Those pachytene spermatocytes that were present were very early types which had developed from leptotene spermatocytes which were obviously not affected at the time of toxicant exposure. In addition, all elongate spermatids were also absent irrespective of

the stage of the spermatogenic cycle. Due to the kinetics of spermatogenesis these elongate spermatids would have been round spermatids at the time of exposure. Since elongate spermatids were identified at all appropriate stages at 3 days post-treatment it would be impossible for elongate spermatid maturation and spermiation to take place in such a short time period (i.e. 96h from 3 to 7 days post-treatment). It is also unlikely that the kinetics of spermatogenesis would have been altered drastically such that step 8 to step 19 elongate spermatids could have matured and been released within 96h and, therefore, the effects of NB were manifested on round spermatids and elongate spermatids between 3 and 7 days after dosing. However it seems unlikely that germ cells perturbed by NB in the round spermatid phase of maturation would have developed into elongate spermatids before degenerating, particularly as degenerating round spermatids were observed 3 days post-treatment.

Pharmacokinetic studies (Rickert *et al*, 1981; 1983) have shown that 50% of a single oral dose of NB is excreted within 24h of administration. Therefore the testicular effects of NB are probably induced within the first 24h after treatment. Thus the degeneration of elongate spermatids and possibly round spermatids may not be mediated directly by NB since these germ cell types degenerated some time after NB has been eliminated. A possible explanation for round and elongate spermatid degeneration is that Sertoli cell function is perturbed in a pronounced but progressive manner. This resulted in the retraction of Sertoli cell cytoplasm, the loss of biochemical and structural maintenance of germ cells and the progressive loss of round spermatids at 3 days and elongate spermatids by 7 days post-treatment. The apparent sensitivity of pachytene spermatocytes to NB (seen in the first 24 to 48h post-treatment) is similar in relation to time and effect to that observed following mDNB administration (Chapter 3) and does not, therefore, exclude the possibility that the Sertoli cell is the target for NB, since Sertoli cell functional changes (i.e. the increased secretion of inhibin into testicular IF and changes in secretion measured *in vitro*) might be manifested by a loss of pachytene spermatocytes. Furthermore, the progressive loss of other germ cells types at 3 and 7 days post-treatment is indirect evidence indicating that NB is a Sertoli cell toxicant which produces pronounced cellular perturbation mediated as germ cell degeneration over a 7 day period post-treatment.

In addition to the histological observations observed over the first 7 days after NB treatment, the levels of serum FSH measured at 3 and 7 days were higher that those at 1 day, reaching a peak at 7 days. Inhibin levels measured in testicular IF at 3 and 7 days post-treatment were also raised

significantly, but to levels equivalent to those measured at 1 day post-treatment. The significant rises in serum FSH levels at 3 and 7 days post-treatment probably reflect the degree of testicular dysfunction, germ cell degeneration and testicular weight reduction observed at these time points. These time-dependent increases in serum FSH levels have also been observed in rats following the administration of other testicular toxicants or following disruption of spermatogenesis experimentally by other chemical and physical means. These testicular toxicants include mDNB (Chapter 3 and Rehnberg *et al*, 1988) and MAA (see Chapter 6 and Bartlett *et al*, 1988) while experimental procedures include cryptorchidism (Jegou *et al*, 1983) and heat treatment (e.g. Bartlett & Sharpe, 1987). These treatments induce substantial germ cell degeneration which is apparent before the increase in serum FSH.

In parallel with raised FSH levels and as a direct consequence of germ cell degeneration, testis weight declines. NB induced a time-dependent decline in testis weight consistent with a significant loss of germ cells within 3 to 7 days of treatment.

Levels of inhibin in testicular IF at 3 and 7 days post-treatment remained elevated to levels equivalent to those measured 24h post-treatment. This seems surprising since serum FSH levels increased at both 3 days and 7 days (in comparison to values observed at 24h), to reach a zenith at 7 days post-treatment. If a correlation between FSH and inhibin exists then why should FSH levels increase while inhibin levels remain significantly elevated but equivalent in value at 1, 3, 7 and 14 days post-treatment, particularly when at 14 days post-treatment serum FSH levels in NB-treated rats were not significantly different from controls? The answer may possibly be because inhibin levels in our experiments have been measured in testicular IF.

Recent evidence suggests that the levels of inhibin in testicular IF often, but not always, change in parallel with peripheral blood levels (Maddocks & Sharpe, 1990). However, the main route by which inhibin reaches peripheral blood in the adult rat is not by secretion into IF but by secretion into seminiferous tubule fluid with subsequent resorption from the rete testis via the overlying mediastinal venous plexus (Maddocks & Sharpe, 1989; 1990).

Changes in the route of secretion of inhibin (i.e. into IF or seminiferous tubule fluid) do occur physiologically during sexual maturation and probably after experimental disruption of spermatogenesis (Maddocks & Sharpe, 1989; 1990). IF levels of inhibin may therefore **not** always parallel the changes found in peripheral blood. Disruption of Sertoli cell function due to NB exposure may therefore have resulted in changes in the route of secretion of

inhibin such that levels in IF were increased. Obviously this problem would be resolved simply by measurement of the peripheral blood levels of inhibin. Unfortunately at the time of the experiment, samples of peripheral blood **serum**, not **plasma**, were collected from NB-treated rats, and it was only subsequently that it was shown that the clotting of blood during serum formation results in the release of unknown factors which cross react in the inhibin RIA (see section 2.3.3.).

Histological observations at 14 days post-treatment revealed that germ cell degeneration had ceased and no further giant bodies were observed. Furthermore, repopulation and expansion of the epithelium was taking place with germ cells up to stage XIV secondary spermatocytes present. This repopulation of the epithelium continued, such that by 35 days post-treatment, all complements of germ cells at all stages of the spermatogenic cycle were present in many seminiferous tubules, although by 21 days post-treatment a small population of tubules was observed in which spermatogenesis had completely failed. Within these tubules only Sertoli cells and spermatogonia were present. This phenomenon has been observed following the administration of a number of other testicular toxicants and quantitatively assessed following mDNB treatment by Hess *et al*, (1988). These authors reported that by 72 days post-treatment, 5 to 20% of tubules were either regressed or in a process of regeneration. It therefore seems likely that, possibly because of cellular damage to the Sertoli cells within these seminiferous tubules, re-initiation of spermatogenesis did not take place. Since the tissue distribution of toxicants such as NB and mDNB is presumed to be equivalent throughout the testis, all Sertoli cells are presumed to be exposed to the same concentration of toxicant. It is entirely possible that Sertoli cells at particular stages of the spermatogenic cycle are more susceptable than others to the adverse effects of NB and the frequency of occurrence of Sertoli cell only tubules could be related to the frequency of duration of certain stages present during toxicant perturbation. However, such a possibility could not explain the synchrony of stages observed after NB-treatment as this was evident before the appearance of Sertoli cell only tubules and was also far more pronounced than could be accounted for by the small percentage of tubules containing only Sertoli cells.

In contrast to the repopulation of the testis with germ cells beyond 14 days post-treatment, testicular weight reached a nadir at 7 days post-treatment and remained at this low level up to 35 days post-treatment. Even by 70 days testicular weight in NB-treated rats was significantly below control levels while histological observations showed quantitatively normal spermatogenesis in most

tubules. The apparent discrepancy between normal spermatogenesis in most tubules but significantly reduced testicular weight may be explained by the presence of Sertoli cell only tubules, although as these were of low frequency, it is also likely that even in the apparently normal tubules, germ cell numbers were quantitatively subnormal.

Levels of serum FSH and inhibin in interstitial fluid remained equivalent to controls at later time points up to 70 days post-treatment. Serum LH levels were only raised significantly at 14 days post-treatment while serum and IF levels of testosterone following NB-treatment were equivalent to controls throughout the experiment. Since no cellular damage or degeneration of Leydig cells could be observed at the histological level following NB administration, it seems likely that any changes which did occur were the result of a general perturbation of testicular function rather than to a direct effect on the Leydig cell and the induction of raised serum LH levels in addition to no effects on Leydig cell testosterone production.

In addition to the germ cell degeneration which occurred over the first 7 days post-treatment, a change in the frequency of occurrence of certain stages of the spermatogenic cycle was observed after NB-treatment. This was initially evident at 3 days post-treatment as an increase in the frequency of stages I to IV, and a decrease in stages XII to XIV. Since spermatogenesis is a precisely timed process, adjacent cellular associations advance to the next stage in wave-like formation (Parvinen, 1982). Because the amount of time that cells remain in a particular association is constant (Courot *et al*, 1970) the frequency of a particular stage in cross sections of the testis is proportional to the percentage of time occupied by that stage (Clermont & Harvey, 1967; Creasy *et al*, 1985). However there is very little information regarding alterations in the duration of the 14 stages of spermatogenesis in the rat (Chapin *et al*, 1984).

A possible explanation for the change in stage frequency observed 3 days after NB treatment was that the maturation of the developing germ cells in these stages had been arrested and/or the duration of spermatogenic stages I to IV had increased over this 72h period post-treatment. The former of these two possibilities seems most likely. Whatever the explanation, for stage synchrony to occur within 3 days, the kinetics of spermatogenesis must have been altered in some way over the first 48 to 72h after NB administration. There are other reports of the kinetics of spermatogenesis being altered by toxicant administration. Russell *et al* (1983) has demonstrated a similar asynchrony of cell types following exposure to procarbazine, but an effect on the frequency of stages was not determined. From 3 days onwards it was possible to predict,

using the sum of the duration of each stage, which stages were increased in frequency. This suggests that the duration of each stage had returned to normal 3 days after treatment such that, by 7 days post-treatment, the increased frequency of occurrence of stages I to IV observed at 3 days was manifest 96h later as a significant increase in the number of tubules in stages V to VI. Therefore the increase in frequency evident at 3 and 7 days post-treatment could be observed histologically and followed chronologically at successive time intervals after NB administration. Significant increases in the frequency of occurrence of certain stages were observed and counted at all time points.

By 70 days post-treatment, the increased frequency of stages I to IV and V to VI observed at 3 and 7 days respectively post-treatment were still evident. During this period almost 6 spermatogenic cycles had taken place, yet this increase was still apparent.

Other compounds reported to cause shifts in the frequency of stages of the spermatogenic cycle include diethylcarbomyl methyl-2,4-dinitropyrrole (Patanelli & Nelson, 1964), heat treatment (15 mins at 43°C) (Chowdhury & Steinberger, 1964), 2-5 hexanedione (Chapin et al, 1982), and ethylene glycol monomethyl ether (Chapin et al, 1984c; Creasy et al, 1985). However, in many of these instances the changes in stage frequency are relatively minor in comparison to those reported here after NB treatment.

Although the precise explanation for stage synchrony remains to be established, it is an interesting phenomenon with considerable application for the study of stage-dependent changes in Sertoli cell function, as has been demonstrated in rats after vitamin A depletion (Huang et al, 1989). Furthermore, if the stage synchrony evident early after NB treatment is due to transient arrest at a particular stage, as a result of some specific disturbance of Sertoli cell function, then it is possible that stage synchrony can be used as a further index of Sertoli cell dysfunction.

Evaluation of the epididymal germ cell content in rats allowed a further, indirect index of testicular germ cell degeneration to be made at successive time intervals after NB treatment. Within 72h of NB administration germ cell types other than sperm were present in the caput region of the epididymis. By 7 days, large giant bodies were present within this region, results similar to those described by Bond et al (1981). Further histological examination of epididymides removed from NB-treated animals at other time points revealed the progression of necrotic testicular germ cell debris through the length of the epididymis. Furthermore, following the progression of this debris through the epididymis, the latter was denuded of sperm and degenerating

germ cells. By 28 days post-treatment, the caput and mid region of the epididymis were devoid of cells while the cauda contained giant bodies and an observable decrease in the density of sperm. However, sperm were not totally absent from the epididymis at all time intervals after treatment, therefore although sperm density per ejaculate was probably significantly reduced, the animal may not have been completely infertile. For an inbred animal such as the rat, sub-fertility would not represent a major problem in reproduction. However in a species such as man with a relatively low fertility rate (by comparison to the rat) a decrease in the normal concentration of sperm in the ejaculate may be very apparent as a significant decrease in fertility rate. This represents one of the major problems for toxicity testing, when doses of toxicant sufficient to cause subfertility in animal species such as the laboratory rat would probably cause a prolonged period of infertility in man.

The histological observations of sperm reserves present within NB-treated rats at these time points however may not give a true reflection of the concentration of sperm present in the epididymis. Sperm reserves would be depleted successively during copulation and, as a result, the animal would probably undergo a period of infertility around 28 days post-treatment. This infertility would last approximately 10 days, allowing for elongate spermatids to mature and be released (at approximately 34 days post NB-treatment) and an epididymal transit time of 7 days. These results are comparable to those observed by Levin *et al* (1988). Following a single exposure to NB (300 mg/kg) and using vasovasostomised rats to continuously monitor sperm output, sperm were not detected in the urine of treated rats between 32 and 48 days post-treatment. They also reported a dramatic increase in sperm and cellular debris in the urine of treated rats at about days 13 to 15. This would correlate exactly with the generation and transit time of giant bodies and sloughed cells from the testis through the epididymis observed in the present study.

The ability of the testis to recover from toxic insult and the animal to progress through a period of subfertility has been reported for NB and other nitroaromatic compounds by other investigators. Treatment of rats with 145 mg/kg of NB for 5 days resulted in a loss of fertility during weeks 3 to 6 in a modified dominant lethal assay. An improvement in fertility was noted in these same rats 6 to 8 weeks after treatment (Rushbrook *et al*, 1985). In an inhalation study using nitrobenzene, a 5- fold increase in the fertility index was reported after a 9- week recovery period (Dodd *et al*, 1987).

Exposure of Sertoli cells in culture to NB resulted within 24h in changes similar to those observed *in vivo*. These included Sertoli cell

vacuolation together with the exfoliation of germ cells and, in addition, dose-dependent increases in the secretion of lactate and pyruvate were observed in the presence or absence of germ cells; the latter effects are similar to those observed *in vitro* for a known Sertoli cell toxicant, mDNB (Chapter 3 and Williams & Foster, 1988). However, the present results suggest that NB is considerably less toxic than mDNB *in vitro* and probably sustantiate the 12- fold difference in dose for a single oral dose of NB (300 mg/kg) required to produce a similar degree of testicular disruption when compared with mDNB (25 mg/kg) administered *in vivo* . While reproducible biphasic changes in inhibin secretion were observed following NB exposure in both culture systems, these effects were different to those of mDNB (which had no effect at the single dose used in this study but effects at different doses - see Chapter 3), and were not evident in FSH-stimulated cultures. The present results therefore suggest that NB produces specific perturbation of Sertoli cell function *in vitro*, similar to other Sertoli cell toxicants, and that the spermatogenic disruption induced by NB *in vivo* is therefore probably the result of a direct effect on the Sertoli cell. The present results observed in culture also suggest that while inhibin secretion may not be a useful marker of the toxic actions of NB on the Sertoli cell *in vivo*, it certainly merits further study using other toxicants.

After exposure to high concentrations ($>5 \times 10^{-4}$M) of NB the *in vitro* secretion of lactate and pyruvate by Sertoli cells was increased in a dose-dependent manner, both in the presence and absence of FSH and in the presence or absence of germ cells. Why the secretion of lactate and pyruvate by Sertoli cells *in vitro* is increased following toxicant administration remains unknown, but it has been demonstrated in a number of cases by agents targeted towards the Sertoli cells *in vivo*, examples of which include mono -2-(ethylhexyl) phthalate (Chapin *et al*, 1988) and mDNB (Williams & Foster, 1988). On the basis of using lactate and pyruvate as indices of Sertoli cell function, the present results suggest that NB induces toxicity to the Sertoli cell via a similar mechanism to mDNB. Further support for this interpretation is provided by the demonstration *in vivo* that administration of NB results in disruptive effects on spermatogenesis similar to those induced by mDNB (Chapter 3 and Foster *et al*, 1986; 1987), involving rapid (1 to 3 days) and widespread exfoliation /degeneration of different germ cell types, consistent with a direct effect of NB on the Sertoli cell.

Previous studies have demonstrated that the testicular toxicity of various compounds observed *in vivo* can be modelled effectively in a Sertoli cell culture system. For example, 2, 5 hexanedione administered *in vivo* causes

spermatogenic disruption in rats (Chapin *et al*, 1982). Further studies *in vitro* demonstrated an increase in lactate secretion from Sertoli cell cultures, which led to the postulation that the cause of testicular toxicity *in vivo* was an effect on the Sertoli cell (Chapin *et al*, 1986). Taking this into account, the pachytene spermatocyte degeneration observed *in vivo* 24h after NB administration may be the result of increased Sertoli cell lactate production. Pachytene spermatocytes are germ cells which are dependent on lactate production to a significant extent, more so than other germ cell types, if only because of their size. However an excess of lactate may be as toxic as a deficiency. Therefore a significant increase in lactate production *in vivo* would be observed in addition to a specific, possibly stage-dependent, degeneration of pachytene spermatocytes.

The present study has also measured an index of Sertoli cell function not previously used to assess toxic action *in vitro*, namely inhibin secretion. Following exposure of co-cultures or Sertoli cell cultures to NB, basal inhibin secretion exhibited a reproducible alteration in response in a biphasic pattern. Although the cause of this pattern is unknown, it was observed in both co-culture and Sertoli cell monocultures; it is therefore unlikely to be due to exfoliation of selective germ cell type(s), a possibility that arises from the probable control of inhibin secretion by specific germ cell types (Pineau *et al*, 1990). Indeed, the consistently higher secretion of inhibin by Sertoli cell-germ cell co-cultures in the present study, when compared with that by Sertoli cell cultures, is further evidence for germ cell modulation of Sertoli cell inhibin secretion. The biphasic pattern of inhibin secretion was, however, not evident under FSH-stimulated conditions and as this situation is more akin to the situation *in vivo*, the present data would not support the use of inhibin as a specific indicator of the toxic actions of NB on the Sertoli cell. Nevertheless, our studies of the effects of NB *in vivo* suggest that increases in inhibin secretion after NB administration are detectable *in vivo* in testicular IF. These significant increases were evident up to 14 days post-treatment and may reflect a prolonged perturbation of Sertoli cells consistent with histological observations of degeneration made *in vivo*. In view of this and the present data for cultures not stimulated by FSH, it is considered that the study of the effects of other testicular toxicants on inhibin secretion *in vitro* and *in vivo* is warranted to establish whether it might yet have potential as a new marker of toxicant action on the testes both *in vivo* and *in vitro*.

mDNB is a known testicular toxicant *in vivo* with overwhelming evidence pointing towards the Sertoli cell as the primary target for its toxicity (see Chapter 3 and Cody *et al*, 1981; Blackburn *et al*, 1985; 1988; Foster *et al*,

1986; Linder *et al*, 1986; 1988; Hess *et al*, 1988). mDNB has also been shown to disrupt Sertoli cell function *in vitro*, causing dose-dependent increases in the secretion of lactate and pyruvate (Williams & Foster, 1988) together with dose-dependent exfoliation of viable germ cells and morphological changes (vacuolation) in Sertoli cells (Chapter 3 and Foster *et al*, 1987). In this study mDNB, at the single dose level used, produced effects on lactate and pyruvate secretion similar to those reported by previous investigators. However, equimolar doses of NB did not produce equivalent increases in the secretion of lactate and pyruvate or in the degree of exfoliation of germ cells in comparison to mDNB. This suggests that mDNB is more toxic than NB *in vitro*, a conclusion supported by studies *in vivo* comparing single oral doses of mDNB or NB. Thus, when mDNB was administered at a dose of 25 mg/kg to rats it produced severe testicular disruption (Chapter 3 and Blackburn *et al*, 1988), whereas the lowest dose of NB required to produce equivalent spermatogenic disruption *in vivo* in rats is approximately 300 mg/kg (Bond *et al*, 1981). Tissue distribution studies of ^{14}C-radiolabelled mDNB demonstrate peak testis concentrations of approximately 10^{-5} M (Foster *et al*, 1986). Lower doses than this have been demonstrated to perturb Sertoli cell function *in vitro* (Williams & Foster, 1988). Calculations based on pharmacokinetic studies of NB metabolism in rats using 300 mg/kg (Rickert *et al*, 1983) suggest that a testicular concentration of approximately 10^{-4} M NB may be produced, although this calculation is based on many assumptions and therefore may be inaccurate. However, on the basis of these calculations there was a 10-fold difference in the intratesticular concentrations of these two toxicants and this is consistent with the *in vitro* differences in sensitivity of the Sertoli cell to NB and mDNB observed in the present study. In contrast to NB, mDNB (at the single dose level used) failed to stimulate basal inhibin secretion in either the presence or absence of germ cells. This could mean mDNB has a different mechanism of toxicity to NB. However, since in all other respects both compounds exert comparable effects *in vitro* (allowing for dose), it is likely that lower doses of mDNB would have resulted in increased basal secretion of inhibin in a manner comparable to that of NB (see Chapter 3 Fig 3.6.).

In summary, the present studies suggest that NB is a testicular toxicant which causes specific disruption of spermatogenesis, with the available evidence suggesting that this probably occurs via a direct effect on the Sertoli cell. These data also support the growing number of studies to have demonstrated that *in vivo* effects of toxicants can be modelled *in vitro* (Gray, 1988) and, in addition, can be used to pinpoint the site of action of the toxicant

in question. Such culture systems can therefore be used to determine the mechanism of action of testicular toxicants at the cellular level. The present study is the first to evaluate the potential of inhibin secretion by Sertoli cells as a marker of toxicant action. The findings suggest that more detailed studies of the effects of a wide range of testicular toxicants on inhibin secretion are warranted to assess whether it might be an alternative indicator of toxicant action on the Sertoli cell to those currently used indices such as lactate and pyruvate, since it has the additional advantage that it can be measured *in vivo* in peripheral plasma.

CHAPTER 5.

MONONITROTOLUENE.

5.1. INTRODUCTION.

The nitrotoluenes are an important industrial group of chemicals. They comprise three structurally related compounds which differ in the number and position of nitro group substitutions on the basic toluene structure. These are the mononitrotoluenes (MNT), dinitrotoluenes (DNT) and trinitrotoluenes (TNT).

The MNT's are intermediates in the production of sulphur and azo dyes as well as rubber and agrochemicals. The estimated production of MNT in the United States in 1980 was 20,000 tons per annum of which 12,500 tons were 2-MNT, 7,000 tons were 4-MNT and 500 tons were 3-MNT (Dunlap, 1981). DNT is an important industrial chemical particularly in the United States. DNT has several applications, primarily in the production of toluene di-isocyanate (TDA), the building block for the manufacture of polyurethane foams, and in the production of explosives and elastomers. It is also an intermediate in dye manufacture and is employed as a gelatinising and waterproofing agent. The production of DNT in the United States in 1983 has been estimated to be greater than 300,000 tons at a value of 650 million dollars (Hartter, 1984). Commercially available DNT has two major constituents, 2,4 DNT (approximately 80%) and 2,6 DNT (approximately 20%). TNT is universally known as an explosive. First prepared by Willbrand in 1863 (Kirk-Othmer, 1972) it was originally mass produced in Germany in 1891 and was used extensively during the first and second World Wars (Zaklan & Villaume, 1978).

The toxicity of DNT and TNT to both animals and man is well documented. However the literature on the toxicity of MNT remains patchy, probably because it is of lesser economic importance. Several investigators have demonstrated that exposure to each of the three isomers of MNT results in toxicity to different organ systems *in vivo*. Adult male Wistar rats administered 2-MNT (200 mg/kg), 3-MNT (300 mg/kg), or 4-MNT (400 mg/kg) 5 days a week for 6 months showed differences in organ and isomer toxicity. All three isomers produced splenic lesions, and in addition, 2-MNT produced nephrotoxicity together with unscheduled DNA synthesis, suggesting that 2-MNT is a hepatocarcinogen in rats (Doolittle *et al*, 1983). Testicular atrophy was also observed after the administration of either 3-MNT and 4-MNT to rats, but not following 2-MNT administration, suggesting that isomer-specific testicular toxicity may exist (Ciss *et al*, 1980).

The signs of acute DNT intoxication include central nervous system and respiratory depression and ataxia. The median lethal doses of the

primary constituents of technical grade DNT, namely 2,4- and 2,6-DNT, when administered to rats are 568 and 535 mg/kg respectively (Ellis *et al*, 1976). The toxic effects of DNT have been described for several different organ systems in the rat. They include haematological changes (low-grade regenerative anaemia) and neuromuscular effects together with cerebral lesions following the administration of 2,4 DNT. Technical grade DNT (76% 2,4 DNT, 20% 2,6 DNT and 4% other isomers) is a liver carcinogen in rats producing a 100% incidence of hepatocellular carcinomas when adimistered at 35 mg/kg/d for 55 weeks (C.I.I.T, 1982). Further studies have indicated that 2,6 DNT was primarily responsible for the carcinogenic activity of technical grade DNT. Other studies have also reported species, sex and metabolism/excretion differences in rats for the toxicity of DNT (Bond *et al*, 1981).

The reproductive toxicity of DNT is well documented in a number of species, particularly the rat (Lee *et al*, 1976) and dog (Lee *et al*, 1979). Studies involving the acute administration of DNT using C.D. rats fed diets containing 2,6-DNT at a dose of 155 mg/kg/d or 2,4-DNT at a dose of 265 mg/kg/d for 30 weeks have demonstrated testicular atrophy and aspermatogenesis with very few germ cells within the epithelium of treated rats at any stage of spermatogenesis. Further studies over a 2 yr period feeding C.D. rats a diet containing 2,4-DNT at a dose of 45 mg/kg/d resulted in spermatogenic disruption (Ellis *et al*, 1978). As a result of this a more complicated three generation study was performed in C.D. rats using doses of 2,4-DNT up to 34 mg/kg/d. Surprisingly, the only adverse reproductive effects observed were associated with the gross toxicity of 2,4-DNT. The lack of effect of 2,4-DNT on reproductive performance in this study (Ellis *et al*, 1979) is most interesting when compared with the adverse histological effects of 2,4-DNT on the testis at higher concentrations. Bloch *et al* (1988) have demonstrated that when 2,4-DNT was fed to adult male rats at 0.2% of the diet for 3 weeks marked vacuolation of the Sertoli cells at the ultrastructural level together with increases in the circulating levels of FSH and LH was evident, in addition to reduced epididymal weight and reduced epididymal sperm reserves. These findings suggest that the initial site of action of DNT may be the Sertoli cell which results in disruption of spermatogenesis through Sertoli cell perturbation and secondary changes in the testicular-pituitary endocrine axis. Further work has been performed using the DNT-derived compound TDA (Varma *et al*, 1988). TDA has been demonstrated to reduce fertility, arrest spermatogenesis and diminish circulating testosterone levels in rats fed 0.03% TDA for 10 weeks. The effects of TDA on ABP production and seminiferous tubule morphology were also

examined. A 7- to 9- fold increase in total testicular ABP content was recorded following 10 weeks of TDA administration in addition to degenerative changes in Sertoli cells observed at the electron microscopic level together with a 50% decrease in epididymal sperm reserves. These effects were reversibile upon removal of TDA administration. Varma and co-workers suggested that the disruption of spermatogenesis occurred within 3 weeks of initial exposure and consisted of a direct effect on the Sertoli cell.

Acute and sub-chronic toxicity experiments involving the administration of TNT to rats have also been conducted. The evaluation of the toxicity of TNT in Fischer 344 rats was preformed using doses up to 300 mg/kg/d in the diet administered over a 90 day period. The reported toxic effects included decreased body weight gain, elevated serum cholesterol levels, anaemia and testicular atrophy consisting of degeneration within the seminiferous epithelium at 125 and 300 mg/kg/d (Levine *et al*, 1984). Histological examination revealed dose-related degeneration of the seminiferous epithelium with, at lower doses, degeneration of primary spermatocytes and round/elongating spermatids in addition to the appearance of giant cell bodies resulting from coalescence of spermatids. At higher doses of TNT, only Sertoli cells and spermatogonia were present in most tubules (Levine *et al*, 1984).

The most economically important of the nitrotoluene derivatives is DNT. Therefore a number of studies have concentrated on the toxicity of DNT in humans. Exposure levels can range from high acute exposure levels (e.g. usually accidental or attempted suicide- MacGee *et al*, 1942) to low level chronic exposure (from either the workplace or from an environmental source, e.g. drinking water). 2,4-DNT is absorbed rapidly through intact skin (Linch *et al*, 1974) and intoxication may also occur due to inhalation or ingestion via the gastrointestinal mucosa (Olishifiski & Mc Elroy, 1971). The major risk of exposure to DNT appears to be greatest for workers in the dye and explosives industries, but environmental contamination may also occur as a result of discharges into rivers and streams from munitions plants (Burrows & Dacre, 1975; Carcinogenic Testing Program, 1978).

There are a number of contradictory studies on the effects of DNT on male reproductive peformance. A number of male workers involved in plastics manufacture have exhibited decreased sperm counts and an increased frequency of abnormal sperm ejaculated following exposure to DNT (N.I.O.S.H., 1980). As early as 1942 the U.S Department of Labour included nitrobenzene (see Chapter 4) and other nitro compounds, (including the nitrotoluenes) in the list of substances considered to be extra hazardous during

pregnancy due to their potential for inducing maternal toxicity (Sullivan & Barlow, 1979). Male reproductive toxicity of 2,4-DNT was also suspected in a number of chemical workers in Kentucky (N.C.D.C. report, 1981). An initial pilot study by the National Institute for Occupational Study and Health (N.I.O.S.H.) Health Hazard Evaluation alleged semen abnormalities, miscarriages and abnormal offspring of employees at the plant, which manufactured DNT and TDA during 1979 and 1980. They found that exposed workers had sperm counts significantly less than controls and abnormalities in sperm structure (Ahrenhalz & Meyer, 1980). However, a more detailed assessment of these workers found normal levels of serum FSH, LH and testosterone together with an abnormally high sperm count in the control groups. This latter study strongly suggested that no reproductive effects existed among male workers exposed to DNT or TDA (Hamill *et al*, 1982).

Human exposure to TNT is usually occupational through its manufacture in munitions factories. The manufacturing by-products can also occur from waste waters discharged from such plants. Occupational exposure to TNT has been reported to occur by inhalation, ingestion and skin absorption. The relative importance of one route of exposure to the others appears to be variable and is probably dependent on the nature of the work operation, the physical state of the material, and the personal hygeine and work practices of the individual employees. Because skin absorption and accidental ingestion of TNT occurs, it is difficult to identify any dose-response relationships for airborne levels of TNT in the workplace. In general, a number of fatalities have occurred following chronic and acute exposure to TNT. The primary sites of toxicity in humans appear to be the liver, kidneys, central nervous system and bone marrow (Hathaway, 1977).

Data on the exposure of humans to MNT is not available probably because it has not been studied in detail in comparison to the di- or tri-nitrotoluenes. This could be due to the relatively unimportant economic role of MNT in comparison to the di- or tri-nitrotoluenes. There are no assessments of male reproductive hazard from occupational exposure to MNT and few assessments of testicular toxicity following acute and sub-chronic oral administration in rats (Ciss *et al*, 1980). Considering the weight of published data reflecting the testicular toxicity of DNT, and to a certain degree TNT, and taking into account the standards and regulations pertaining to the use of these chemicals in the work environment, it is difficult to understand why the testicular lesions produced in rats following MNT administration have not been

further investigated and extrapolated into field studies in the workplace as is the case with di- and tri- nitrotoluene.

The precise nature of the testicular lesion induced by MNT is unknown: Its possible target site within the testis, i.e. the Sertoli cell ? The mechanism of action, i.e. through a direct effect on testicular cells or disruption of the pituitary-testicular hormonal axis ? The compound's possible stage specificity and/or reversibility of any testicular disruption or metabolism of the compounds to identify if the parent compound or a metabolite(s) may be the testicular toxicant are unknown. Ciss and co-workers in 1980 reported testicular effects associated with a decrease in testicular weight coupled with testicular atrophy observed at the histological level using both sub-acute and chronic oral doses in the rat. No experimental data to date either *in vivo* or *in vitro* has taken this work further. Indeed the isomers of MNT may present a further biochemical tool with which to study testicular function and possibly the role of the Sertoli cell and its secretions in spermatogenesis. Therefore, in this study 2-, 3- or 4-MNT have been administered to rats at doses reported to result in testicular disruption (Ciss *et al*, 1980). At intervals ranging from 6 hrs to 5 days after treatment, possible temporal changes in testicular morphology and function have been observed in addition to alterations in hormonal profiles post-exposure. Concomitant with these studies *in vivo*, the effects of 2-, 3- and 4-MNT *in vitro* have been assessed using Sertoli cell cultures and co-cultures together with a number of indices of function including morphology, the exfoliation of germ cells in co-culture and the secretion of inhibin, a possible marker of Sertoli cell perturbation *in vitro*. These cultures were used in order to investigate if previously reported findings of testicular disruption induced by some isomers of MNT *in vivo* (Ciss *et al*, 1980) could be reproduced and investigated in further detail *in vitro* using a rat Sertoli cell culture system.

5.2. METHODS.

5.2.1. Methods for assessment of the effects of the isomers of mononitrotoluene *in vivo*.

5.2.2. Reagents.

The *ortho* (2) *meta* (3) or *para* (4) isomers of mononitrotoluene (MNT) were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K). Analysis by gas chromatography/mass spectrometry before use found 2, 3 or 4 MNT to be 97, 98 and 95% pure respectively. The vehicle for MNT

administration was corn oil (Mazola) obtained from a supermarket. The corn oil was stored under nitrogen gas to prevent its oxidation.

5.2.3. Animals.

A number of studies were performed using rats of either the Alpk: AP (Wistar derived) strain from the I.C.I. Animal Breeding Unit, Alderley Park, Macclesfield, Cheshire, U.K. or of the Sprague Dawley derived strain bred in the Centre for Reproductive Biology in Edinburgh, UK.

5.2.4. Experimental Protocol.

In all studies adult male rats (80 - 120 days old) were allocated randomly in equal numbers (usually 6) per cage and maintained under normal animal house conditions : (12h:12h light dark cycle, 21°C and 35-60% humidity throughout the course of the experiment). Food and water were available *ad libitum*.

A number of *in vivo* experiments were performed in which different doses of compounds were administered by oral gavage in single or multiple dose regimes. At the beginning of each experiment (time 0) rats were administered 2-, 3- or 4-MNT by oral gavage: (a) At a single dose of 250, 500, 750 or 1000 mg/kg bodyweight and killed by an overdose of halothane anaesthetic 120h after treatment. The testes, epididymides, liver, spleen and kidneys were removed from each animal and weighed, and a blood sample was collected from each animal immediately after death for hormone measurement (see section 2.3.). (b) Three oral doses of 250, 500 and 750 mg/kg bodyweight administered every 24h for 72h, and the animals killed by an overdose of halothane anaesthetic 48h after the final treatment (i.e. 120h from time 0). The testes, epididymides, liver, spleen and kidneys were removed from each animal, weighed and a blood sample collected immediately after death for hormone measurement (see section 2.3.). (c) At a single oral dose of 750 mg/kg bodyweight and the animals anaethetised and perfusion fixed at 6, 12, 24, 72 and 120h post-treatment. Testicular and epididymal tissues were removed, weighed and taken for histological examination.

In each experiment control groups received a single or multiple dose of vehicle alone (corn oil) at a dose(s) of 5 ml/kg

5.2.5. Methods for assessment of the effects of the isomers of mononitrotoluene *in vitro.*

5.2.6. Reagents.

For the purity of 2-, 3- or 4-MNT see section 5.2.2. All culture materials were obtained from Flow Laboratories (Irvine, Scotland, U.K.) or Gibco (Paisley, Scotland, U.K). All other biochemical reagents were obtained from Sigma, B.D.H. (Poole, Dorset, U.K.) or Boehringer Mannheim (Lewes, East Sussex, U.K.) and were of the highest purity available.

5.2.7. Animals.

Rats were of the Alpk:AP (Wistar derived) strain from the I.C.I. Animal Breeding Unit, Alderley Park, Macclesfield, Cheshire, U.K.

5.2.8. Experimental Protocol.

5.2.9. Preparation of Testicular Cell Cultures.

Co-cultures were prepared using a modification of the method of Gray & Beamand (1984). Sertoli cell only cultures were prepared from co-cultures using the method of Galdieri *et al* (1981) (see section 2.5.1.).

5.2.10. Treatment of cultures.

Toxicants were administered to cultures 72h after plating. 2-, 3- or 4-MNT were administered at final concentrations ranging between 10^{-7} and 5×10^{-3}M. Toxicants were administered over a 24h incubation period and were applied as solutions in dimethylsulphoxide (DMSO), final concentration 0.3% v/v, while control cultures received an equal volume of this vehicle. Treatments caused no appreciable alteration of culture medium pH. All experiments were performed at least twice, in each case with a minimum of six replicates for each dose and treatment group.

5.2.11. Parameters measured.

Medium was removed from cultures 24h after toxicant administration for the measurement of inhibin (see section 2.3.3.) or for quantitation of germ cell exfoliation (see section 2.5.8.). The remaining cells in each well were used for protein estimation (see section 2.5.8.). Cultures containing coverslips were stained using haematoxylin and Papanacolaou's stain for histological examination (see section 2.5.2.).

5.3. RESULTS

5.3.1. Effect of a single or multiple dose(s) of either 2-, 3- or 4-MNT on testicular and epididymal weight.

A dose-dependent reduction in testicular weight was only observed in rats administered a single or multiple oral dose(s) of 2-MNT in comparison to respective vehicle treated controls (Fig 5.1). Single doses of the 3- or 4- isomers had no significant effect on testicular weight, but multiple doses of either 3- or 4-MNT at the highest dose administered (750 mg/kg/dose) also resulted in significant reductions in testicular weight. In contrast, a significant increase in testicular weight was observed following the administration of multiple doses of 3-MNT at 500 mg/kg/dose (Fig 5.1).

Dose-dependent increases in epididymal weight were observed following the administration of 3-MNT at single oral doses of 250, 500 and 750 mg/kg (Fig 5.1). In addition, a single oral dose of 2-MNT at 1000 mg/kg induced a significant decrease in epididymal weight. Following the multiple administration of different doses of either 2-, 3- or 4-MNT no significant differences in epididymal weight were observed in comparison to respective controls (Fig 5.1)

5.3.2. Effect of a single or multiple oral dose(s) of either 2-, 3- or 4-MNT on liver, spleen and kidney weight.

A significant increase in liver weight was observed after a single oral dose of 2-MNT at 1000 mg/kg while significant decreases in liver weight were observed after single oral doses of 3-MNT at concentrations in excess of 500mg/kg in comparison to respective controls (Fig 5.2). Following a single oral dose of either 2-, 3- or 4-MNT no significant differences in spleen or kidney weight were measured in comparison to respective controls (Fig 5.2).

Following the administration of multiple doses of 4-MNT significant decreases in spleen weight (at all doses), kidney weight (at 750 mg/kg) and a dose-dependent decrease in liver weight were observed (Fig 5.2). While the weights of kidney, spleen and liver fluctuated markedly between animals following multiple doses of all toxicants at all concentrations, significant differences in the weight of these organs were only observed following the administration of 4-MNT (Fig 5.2).
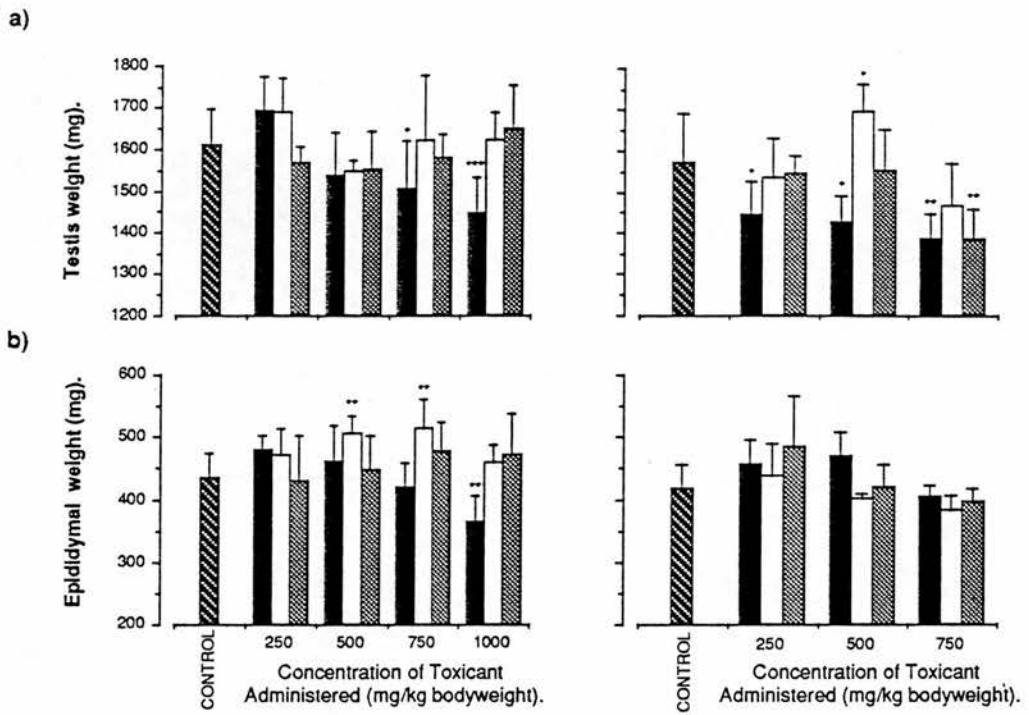
Fig 5.1. Dose-related changes in testicular (**a**) or epididymal (**b**) weight 5 days after a single (left column) or multiple (right column) oral dose(s) of 2 mononitrotoluene (■), 3 mononitrotoluene (□), 4 mononitrotoluene (▨) or control vehicle (▨). Values are means ± SD for 6 rats per group (*P<0.05,**P<0.01,***P<0.005, in comparison to respective controls).

a)

b)

c)
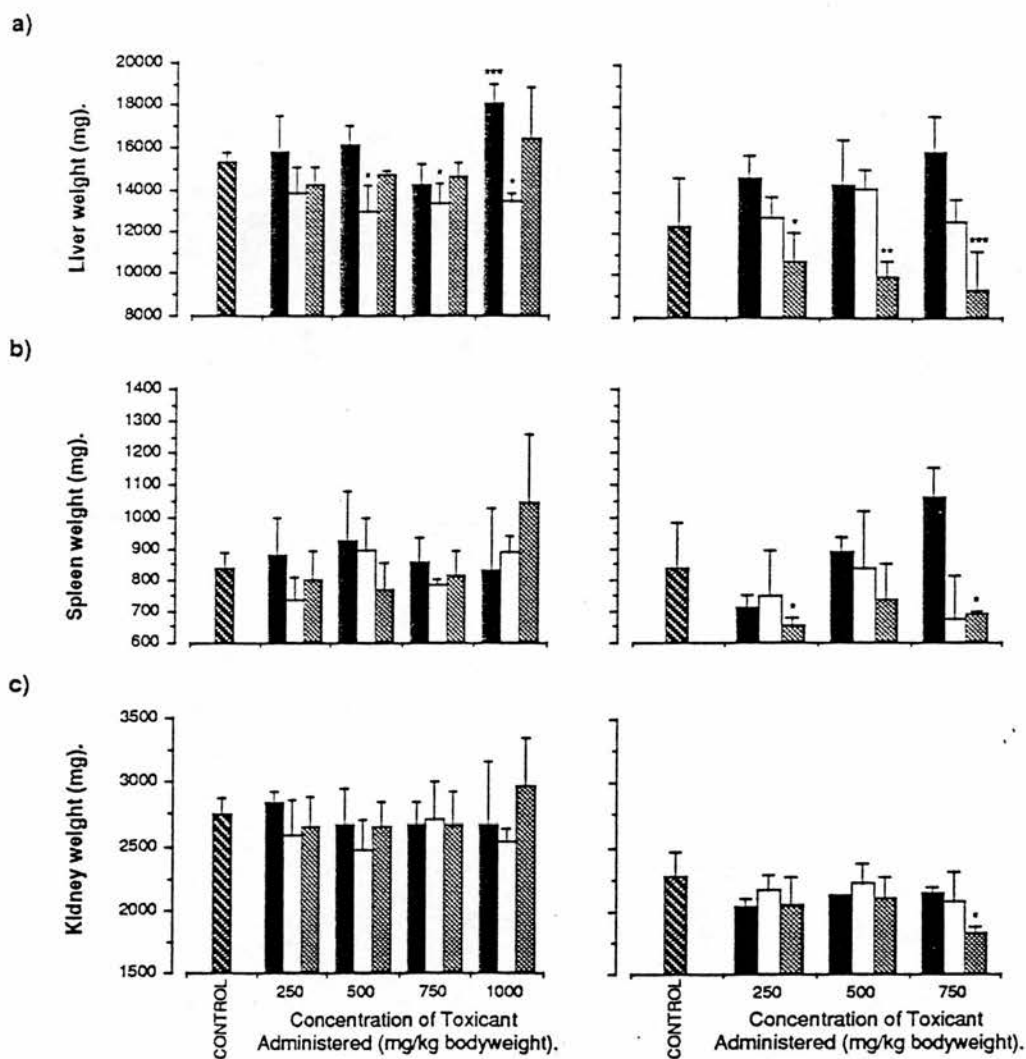
Fig 5.2. Dose-related changes in weights of liver (**a**), spleen (**b**) or kidney (**c**) 5 days after a single (left column) or multiple (right column) oral dose(s) of 2 mononitrotoluene (■), 3 mononitrotoluene (□), 4 mononitrotoluene (▨) or control vehicle (▨). Values are means ± SD for 6 rats per group (*P<0.05,**P<0.01,***P<0.005, in comparison with respective controls).

5.3.3. Effect of a single or multiple oral dose(s) of either 2-, 3- or 4-MNT on serum levels of FSH, LH and testosterone.

A dose-dependent decrease in serum FSH was observed 5 days after the single oral administration of 2-MNT while, in contrast, the reverse was observed following the administration of 3-MNT (Fig 5.3). However a significant decrease in serum FSH levels was only observed after the single oral administration of 2-MNT at a concentration of 1000 mg/kg while significant increases in serum FSH were observed following the administration of 3-MNT at doses of 750 and 1000 mg/kg (Fig 5.3). Multiple doses of 2- or 3-MNT at all concentrations used had little or no effect on serum FSH levels (Fig 5.3). Single oral doses of 4-MNT also had little effect on serum FSH levels at all concentrations used, however multiple doses significantly raised serum FSH levels at doses of 500 and 750 mg/kg/dose in a dose-dependent manner (Fig 5.3).

Although the levels of serum LH and testosterone in rats following a single or multiple doses of either 2-, 3- or 4-MNT fluctuated markedly between animals, no significant differences were observed in comparison with respective controls (Figs 5.4 and 5.5 respectively). In general, changes in serum FSH levels were parallelled by similar changes in serum testosterone levels and to a lesser extent serum LH levels (with the exception of multiple doses of 4-MNT), but because of the between animal variation in serum testosterone and LH levels no consistent or dose-related pattern to these results were observed (Fig 5.4 and 5.5).

5.3.4 Temporal changes in testicular or epididymal weight following a single oral dose of either 2-, 3- or 4-MNT.

Significant decreases in testicular weight were observed after the administration of a single oral dose of either 2- or 3-MNT at a number of time intervals after dosing (Fig 5.6.). In general, a small but significant time-dependent decline in testicular weight was observed following the administration of either 2- or 3-MNT in comparison to grouped control values (Fig 5.6)

In addition, significant decreases in epididymal weight were observed after the administration of a single oral dose of either 2-, 3- or 4-MNT at a number of time points (Fig 5.6). The most significant reductions were observed 120h after the administration of 2- or 3-MNT.
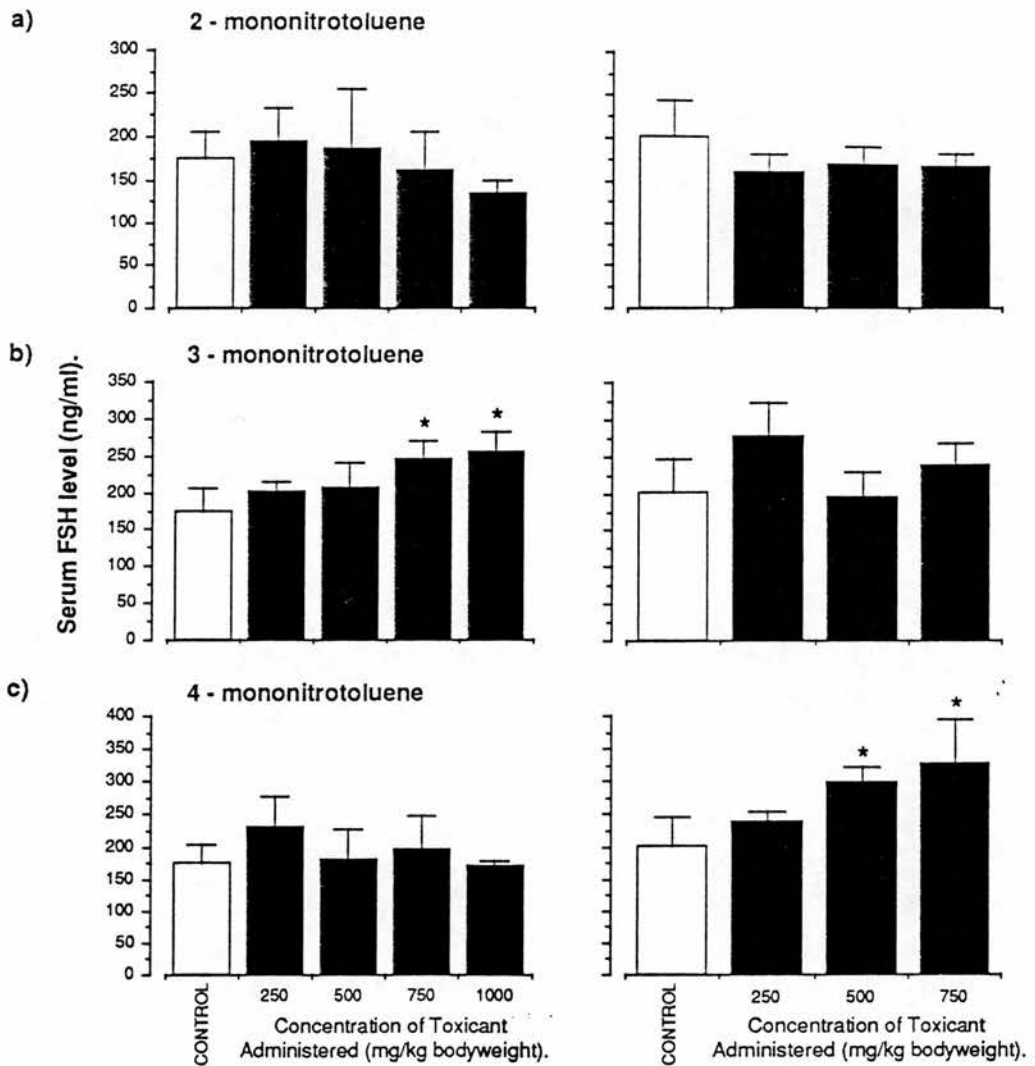
Fig 5.3. Dose-related changes in serum levels of FSH in rats 5 days after a single (left column) or multiple (right column) oral dose(s) of 2 mononitrotoluene (**a**), 3 mononitrotoluene (**b**), 4 mononitrotoluene (**c**) or control vehicle. Values are means ± SD for 6 rats per group (*P<0.05, in comparison with respective controls).
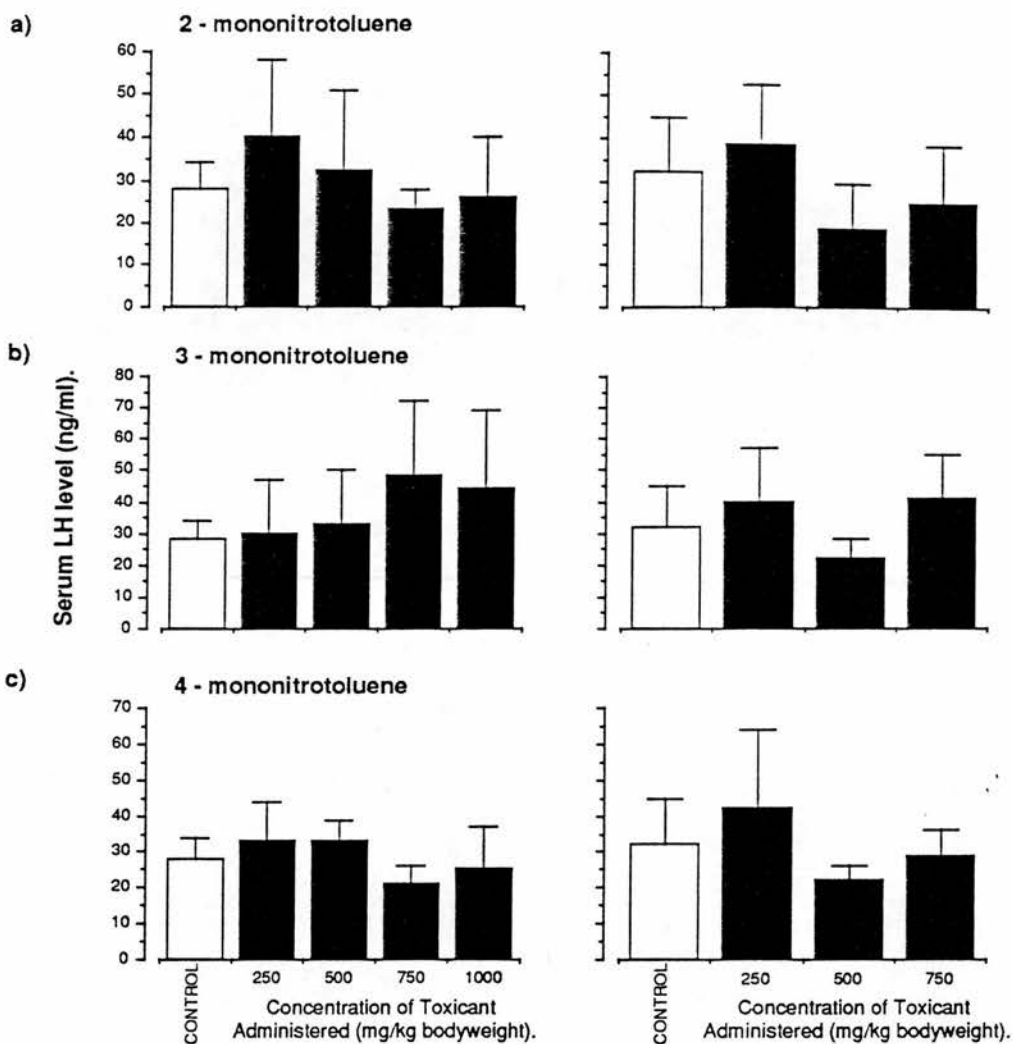
Fig 5.4. Dose-related changes in serum levels of LH in rats 5 days after a single (left column) or multiple (right column) oral dose(s) of 2 mononitrotoluene (a), 3 mononitrotoluene (b), 4 mononitrotoluene (c) or control vehicle. Values are means ± SD for 6 rats per group. No significant differences (P>0.05) were observed.
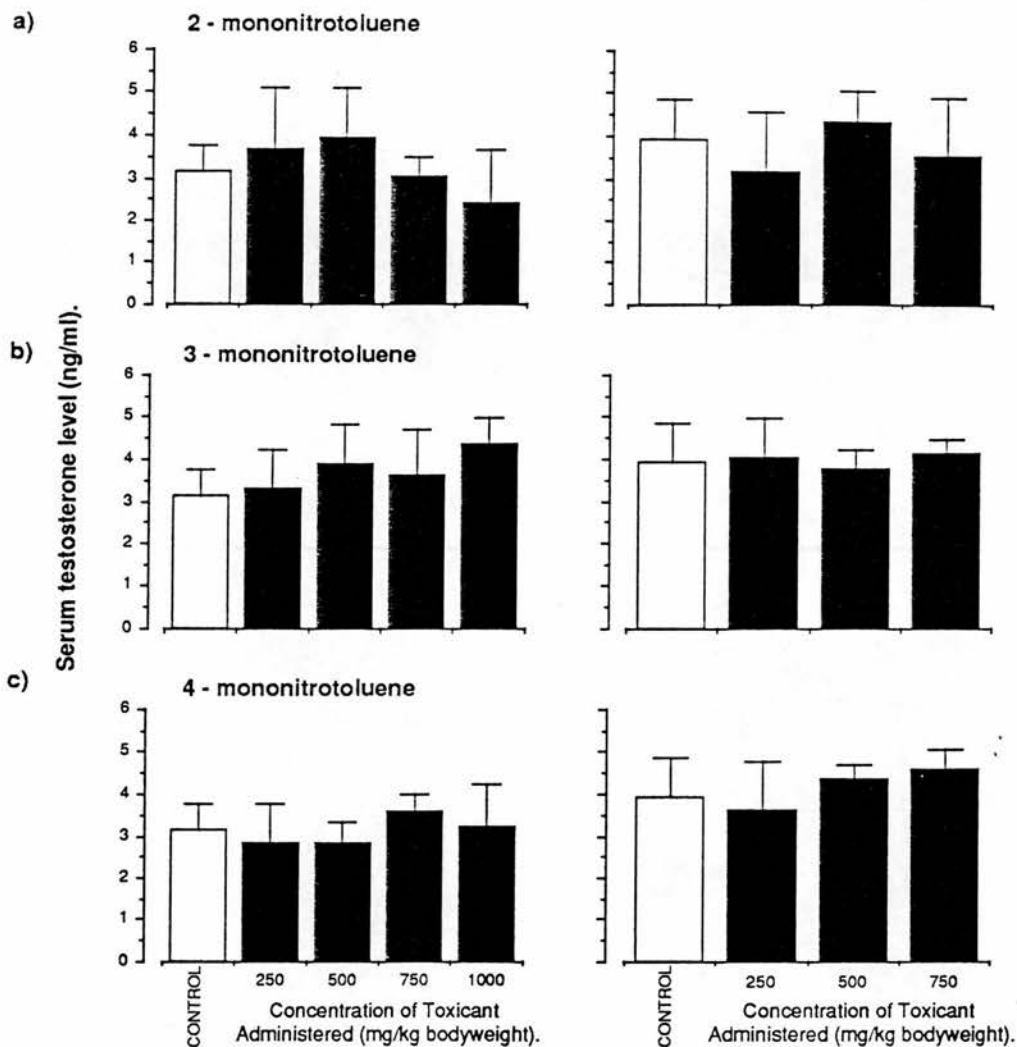
Fig 5.5. Dose-related changes in levels of serum testosterone in rats 5 days after a single (left column) or multiple (right column) oral dose(s) of 2 mononitrotoluene (a), 3 mononitrotoluene (b), 4 mononitrotoluene (c) or control vehicle. Values are means ± SD for 6 rats per group. No significant differences (P>0.05) were observed.

a)

b)
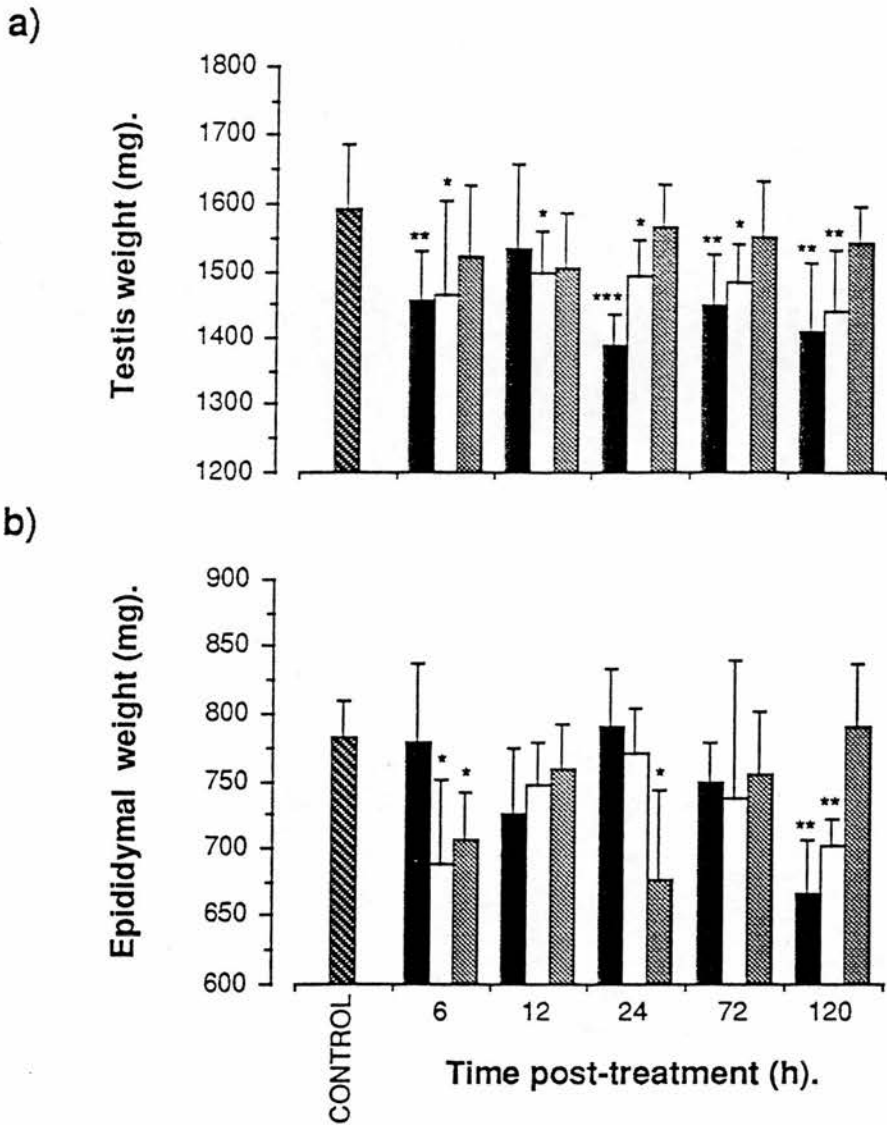
Fig 5.6. Temporal changes in testicular (a) or epididymal (b) weight in perfused tissue after a single oral dose of 2 mononitrotoluene (■), 3 mononitrotoluene (□), 4 mononitrotoluene (▨) at a dose of 750 mg/kg bodyweight or control vehicle (▨). Values are means ± SD for 3 rats per group except for controls, which consisted of 10 rats (2 per time point), (*P<0.05,**P<0.005,***P<0.001, in comparison to respective controls).

5.3.5. Temporal changes in testicular morphology induced by a single oral dose of either 2-, 3- or 4-MNT.

Perfusion fixation was successfully performed on a number of rats at 6, 12, 24, 72 and 120h after the administration of 2-, 3- or 4-MNT. In addition 2 control animals, (administered corn oil alone) were perfused at each time interval. The quality of fixation, processing and staining however were such that it was difficult to distinguish between compound-induced effects and fixation/processing artifact. For reasons which are unclear, in this instance, fixation was sub-optimal and as any effects of the toxicants on the testis were clearly quite minor, they could not be distinguished with certainty from fixation artifact.

5.3.6. Effect of either 2-, 3- or 4-MNT on Sertoli cell morphology and germ cell exfoliation in co-culture.

Control cultures of Sertoli cells and Sertoli cell-germ cells were considered to be normal under histological examination 24h after the administration of DMSO to the cultures (plates 5.1. a, 5.2. a and 5.3. a). Confluent monolayers of Sertoli cells were formed in culture within 3 days of plating. Co-cultures appeared normal with no apparent germ cell necrosis or significant cellular exfoliation of germ cells or Sertoli cells induced by the vehicle (plates 5.1. a, 5.2. a and 5.3. a).

Sertoli cell vacuolation coupled with an observable decrease in the density or absence of different populations of germ cells was evident from visual examination of the co-cultures exposed to 2- or 3-MNT at doses up to 10^{-3}M in comparison to controls (see plates 5.1 b, c, d and 5.2 b, c and d respectively). Although a degree of Sertoli cell vacuolation was observed following treatment with 4-MNT, a visible decrease in the density of different populations of germ cells was apparent from visual examination of the co-cultures exposed to 4-MNT at doses of 10^{-5}M (plate 5.3 b), 10^{-4}M (plate 5.3 c) and 10^{-3}M (plate 5.3. d).

Following exposure to 2- or 3-MNT for 24h a dose-dependent exfoliation of cells from co-cultures was observed (Fig 5.7). These were considered to be primarily germ cells since treatment of co-cultures with 10^{-3}M 2- or 3-MNT resulted in significant exfoliation of cells ($434 \times 10^{-3} \pm 52 \times 10^3$ and $383 \times 10^3 \pm 30 \times 10^3$ total cells/ well respectively), whereas an insignificant number of exfoliated cells ($3.7 \times 10^3 \pm 1.2 \times 10^3$ total cells/well) occurred after exposure of Sertoli cell only cultures to an identical dose of either 2- or 3-MNT. This was presumed to represent the detachment of the residual germ cells that contaminate Sertoli cell cultures following Tris treatment.
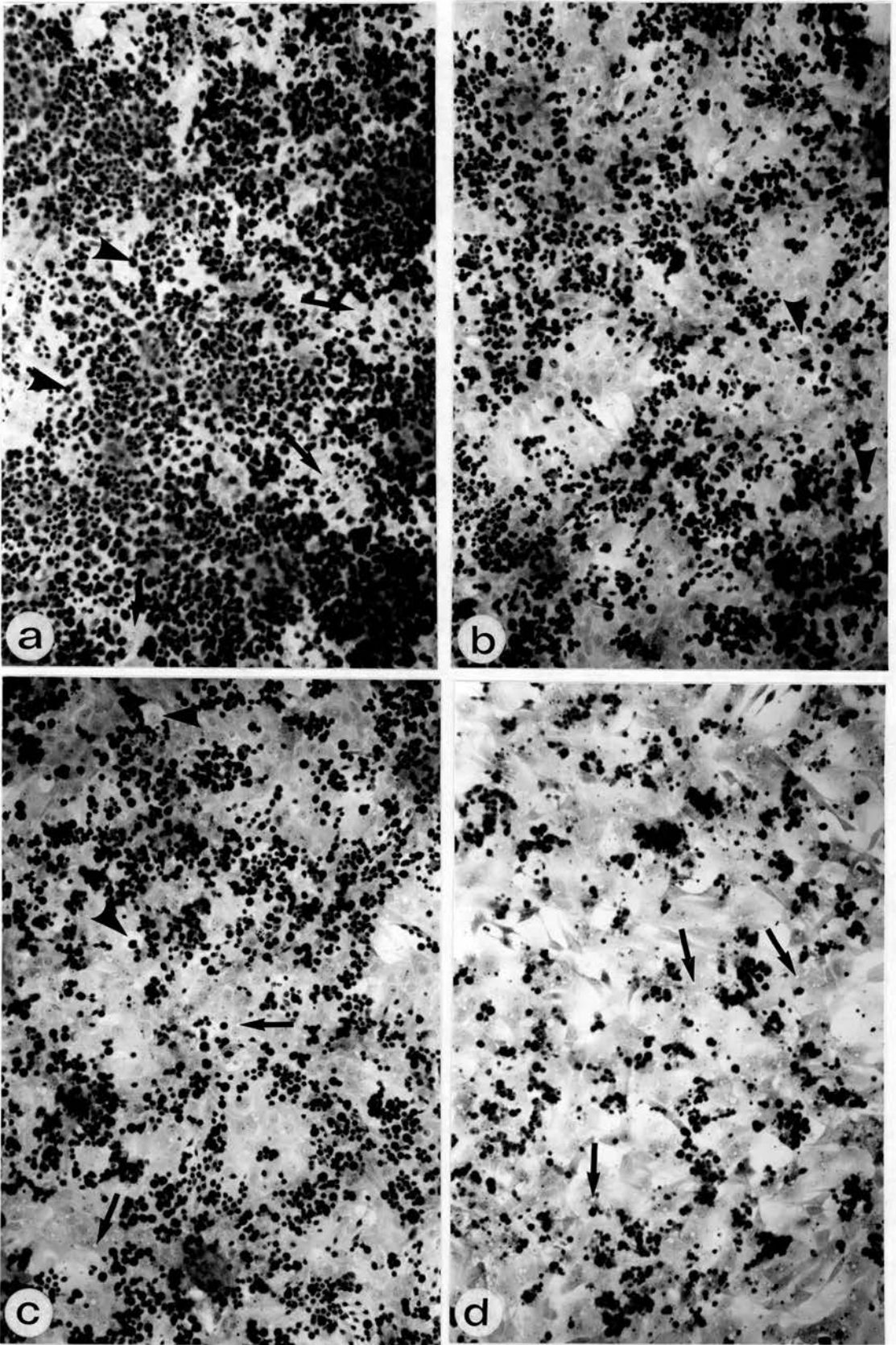
Plate 5.1. Sertoli-germ cell co-cultures following exposure for 24h to (a) control vehicle (DMSO-0.3% v/v), showing confluent Sertoli cells (arrows) beneath numerous adherent clusters of germ cells (arrowheads); (b) 5×10^{-5}M 2-MNT, showing a noticeable decrease in the density of germ cells together with phagocytosis of degenerating germ cells by Sertoli cells (arrowheads); (c) 10^{-3}M 2-MNT, illustrating vacuolation of Sertoli cells (arrows) in addition to phagocytosis of degenerating germ cells (arrowheads); (d) 5×10^{-3}M 2-MNT showing a breakdown in the confluent Sertoli cell monolayer in addition to vacuolation of Sertoli cells (arrows); note the extensive loss of adherent germ cells. Haematoxylin and Papanicolaou's (x75).
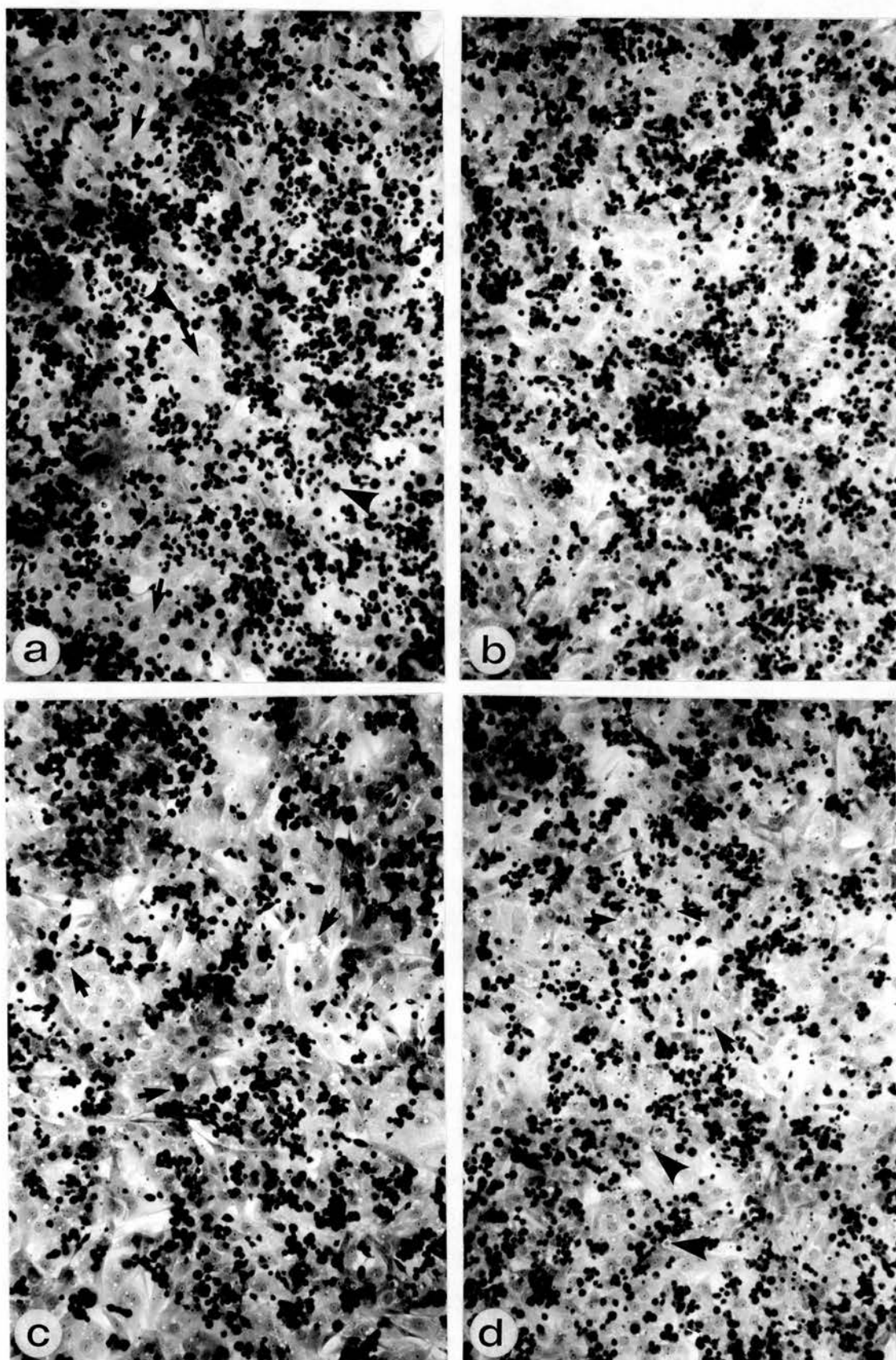
Plate 5.2. Sertoli-germ cell co-cultures following exposure for 24h to (a) control vehicle (DMSO-0.3% v/v), showing confluent Sertoli cells (arrows) beneath numerous adherent clusters of germ cells (arrowheads); (b) 5×10^{-5}M 3-MNT, showing a slight decrease in the density of germ cells; (c) 10^{-4}M 3-MNT, illustrating vacuolation of Sertoli cells (arrows) and a noticeable loss of adherent germ cells; (d) 10^{-3}M 3-MNT showing phagocytosis of degenerating germ cells (arrowheads) in addition to vacuolation of Sertoli cells (arrows). Haematoxylin and Papanicolaou's (x75).
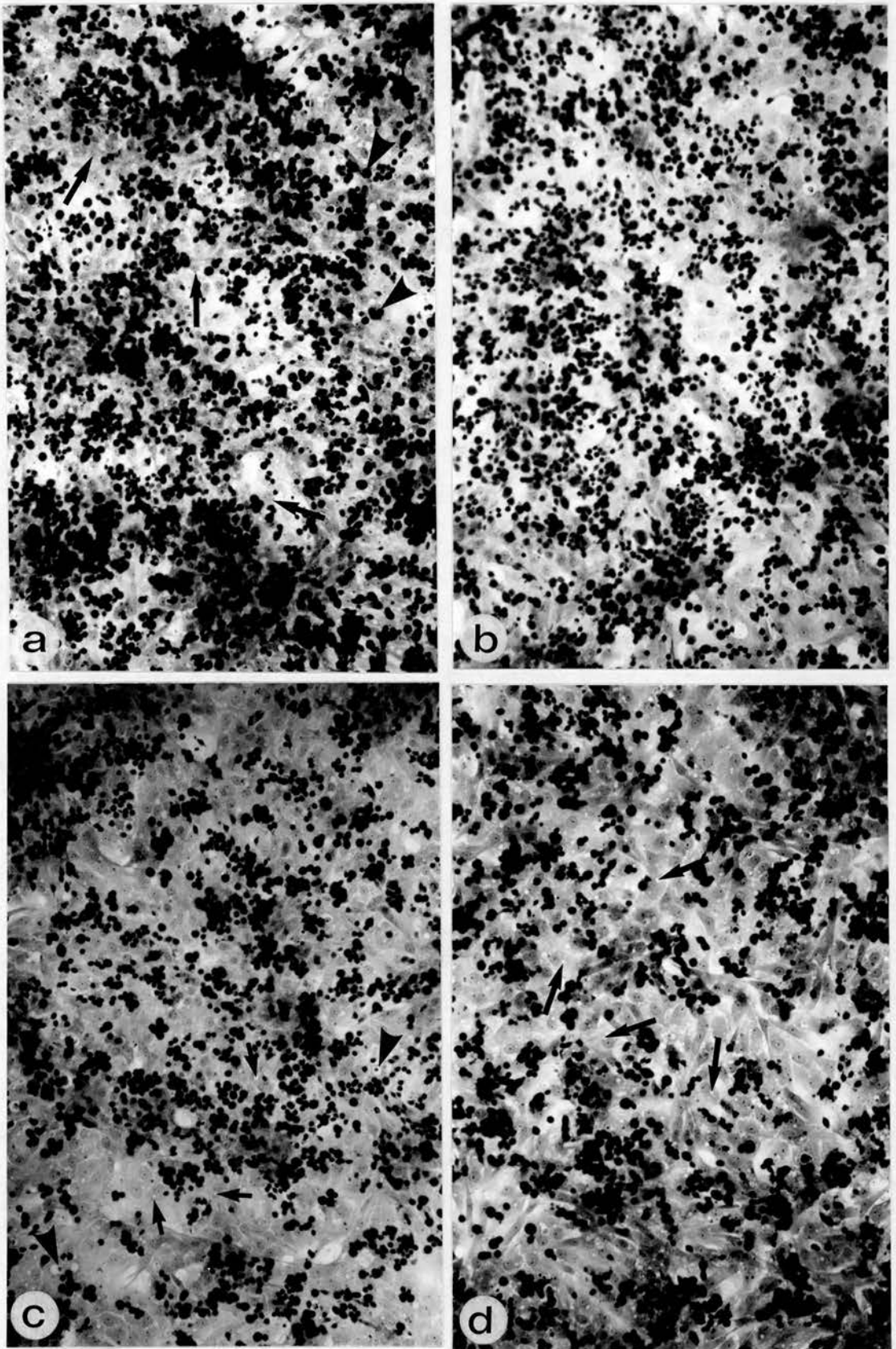
Plate 5.3. Sertoli-germ cell co-cultures following exposure for 24h to (a) control vehicle (DMSO-0.3% v/v), showing confluent Sertoli cells (arrows) beneath numerous adherent clusters of germ cells (arrowheads); (b) 10^{-5}M 4-MNT, showing a noticeable decrease in the density of germ cells; (c) 10^{-4}M 4-MNT illustrating vacuolation of Sertoli cells (arrows), phagocytosis of germ cells by Sertoli cells (arrowed heads) and a loss of adherent germ cells; (d) 10^{-3}M 4-MNT showing extensive Sertoli cell vacuolation (arrows) and germ cell loss. Haematoxylin and Papanicolaou's (x75).

Exposure of co-cultures to 2-MNT resulted in greater exfoliation of germ cells in comparison to 3- or 4-MNT at equimolar doses, while 3-MNT induced greater exfoliation of germ cells than did 4-MNT at equimolar doses (Fig 5.7). Control co-cultures treated with vehicle alone (DMSO) showed significant ($P<0.01$) increases in the detachment of cells when compared to non-treated co-cultures.

In DMSO treated co-cultures, $94 \pm 3\%$ (mean $\pm$ SD) of exfoliated cells excluded trypan blue, indicating that they were essentially viable. After exposure to 10^{-4}M 2-MNT or 10^{-3}M 3-MNT the viability of exfoliated cells was similarly high (83 to 86% and 78 to 82% respectively), but decreased marginally with 2-MNT at 10^{-3}M ($74 \pm 6\%$) and decreased substanially after exposure of co-cultures to 2- and 3-MNT at concentrations of 5×10^{-3}M ($62 \pm 7\%$ and $59 \pm 5\%$ respectively) - see Table 5.1. below. In addition, cells exfoliated after exposure of co-cultures to 4-MNT at a concentration of 5×10^{-3}M also showed increased non-viability (only $66 \pm 5\%$ of cells excluding trypan blue). Thus 2-, 3- or 4-MNT at concentration of 5×10^{-3}M in co-culture were considered to be cytolethal for germ cells.

Table 5.1 The effect of a range of doses of 2-, 3- and 4-MNT on the viability of exfoliated cells removed from the medium of co-cultures 24h after compound administration. Cell counts were performed using a haemocytometer and non-viable cells were identified using 0.05% trypan blue solution; cells which absorbed trypan blue were considered viable. Values are means $\pm$ SD for six replicate cultures (*$P<0.01$,**$P<0.001$, in comparison to control). Results of a representative experiment are shown.

Final concentration of toxicant (M)	Percentage of exfoliated cells absorbing trypan blue dye (mean $\pm$ SD). Compound administered:		
	2 MNT	3MNT	4MNT
10^{-7}	5 ± 1	4 ± 4	5 ± 3
10^{-6}	10 ± 6	8 ± 1	4 ± 6
10^{-5}	13 ± 6	11 ± 3	7 ± 1
10^{-4}	17 ± 3*	14 ± 4	6 ± 5
10^{-3}	26 ± 6**	22 ± 4**	12 ± 4
5×10^{-3}	38 ± 7**	41 ± 5**	34 ± 5**
Control Vehicle DMSO (0.3% v/v)	6 ± 3		
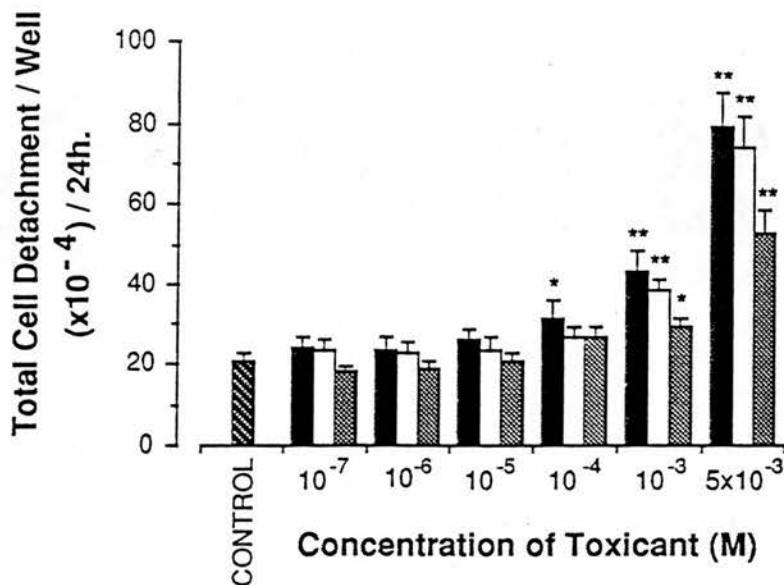
Fig 5.7. Effect of a range of doses of 2 mononitrotoluene (■),
3 mononitrotoluene (□), 4 mononitrotoluene (▨) or control vehicle
(DMSO 0.3% v/v) (▨) on the number of detached cells present in the medium
of Sertoli cell-germ cell co-cultures after a 24h exposure period. Values are
means ± SD for 6 replicates (*P<0.01, **P<0.001, in comparison to control).
Results of a representative experiment are shown.

5.3.7. Effect of either 2,- 3- or 4-MNT on inhibin secretion by Sertoli cells in mono-culture and co-culture.

Significant increases in inhibin secretion were observed after exposure of Sertoli cell-germ cell co-cultures to 2-MNT at concentrations in excess of 10^{-4}M; these levels reached a peak at 10^{-3}M (Fig 5.8). An increase in inhibin secretion was also observed in Sertoli cell cultures exposed to 2-MNT but only at a dose of 5×10^{-3}M in comparison to respective controls (Fig 5.8). In addition, the exposure of Sertoli cells to 3-MNT at a concentration of 10^{-3}M also significantly increased inhibin secretion in the presence or absence of germ cells (Fig 5.8). The addition of 4-MNT at a concentration of 5×10^{-3}M to Sertoli cell cultures or co-cultures significantly decreased inhibin secretion in comparison to respective controls. The administration of 2-MNT to co-cultures at a dose of 5×10^{-3}M stimulated inhibin secretion to the greatest extent.

In all control cultures and co-cultures or in the presence of toxicants at concentrations less than 10^{-4}M the magnitude of inhibin secretion was consistently greater in Sertoli cell-germ cell co-cultures than in cultures of Sertoli cells only (Fig 5.8).

5.4. DISCUSSION.

The present study is the first to evaluate the effects of high single or multiple oral doses of either 2-, 3- or 4-mononitrotoluene (MNT) as systemic and/or testicular toxicants and to correlate changes in the latter to alterations in the levels of serum hormones. Testicular and epididymal weight have been used as indices of testicular dysfunction while spleen, liver and kidney weight have been used as indices of splenic, hepatic and nephrotoxicity respectively. Serum hormones measured included FSH, LH and testosterone. The present *in vitro* studies are also the first to examine the effects of 2-, 3- or 4-MNT on the Sertoli cell *in vitro* using known indices of Sertoli cell function including morphology and germ cell exfoliation (Gray & Beamand, 1984). In addition, Sertoli cell inhibin secretion in the presence or absence of germ cells was used as a further measure of Sertoli cell function *in vitro*.

Previous investigations have centred on the systemic and testicular toxicity of other structurally related toluene derivatives including the isomers of dinitrotoluene (Bond *et al*, 1981; Rickert *et al*, 1981; 1984; Price *et al*, 1985; Lane *et al*, 1985; Bloch *et al*, 1988) and trinitrotoluene (Dilley *et al*, 1982; Levine *et al*, 1984). These extensive studies over the past decade or so have demonstrated that both di- and tri-nitrotoluene are systemic and testicular toxicants inducing hepatic, splenic and nephrotic lesions in addition to varying
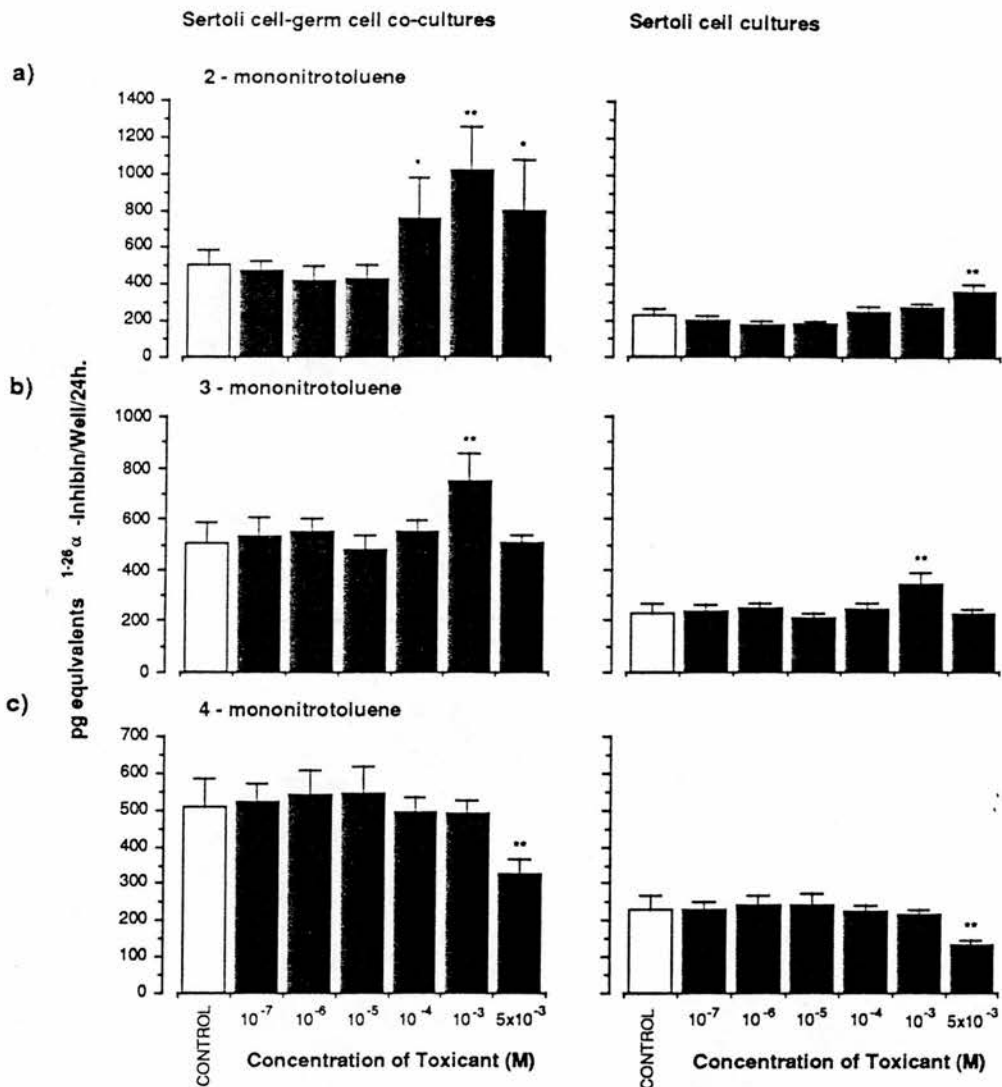
Fig 5.8. Effect of a range of doses of 2 mononitrotoluene (a),
3 mononitrotoluene (b), 4 mononitrotoluene (c) or control vehicle (DMSO
0.3% v/v) (□) on the secretion of $^{1-26}\alpha$-inhibin by Sertoli cell-germ cell
co-cultures (left column) or Sertoli cell monocultures (right column) after a
24h exposure period. Values are means ± SD for 6 replicates (*P<0.05,
**P<0.01, in comparison to respective controls). Results of a representative
experiment are shown. Note however that the Sertoli cell cultures and
co-cultures are derived from separate cell preparations.

degrees of testicular atrophy depending on the dose administered. In addition to these animal tests, epidemiological studies on human males exposed to trinitrotoluene, dinitrotoluene or toluene diamine (an intermediate in the production of polyurethane plastics) suggest that exposure to these compounds in the workplace may result in decreased fertility (Hathaway 1977; Hamill *et al,* 1982). However, while there are a number of investigations into the hepatic and metabolic dysfunctions induced by the isomers of MNT (Kedderis & Rickert, 1984; Chism *et al,* 1984) very few studies have concentrated on their potential testicular toxicity. Since these compounds are chemically similar to di- and tri-nitrotoluene it must be assumed that, like the structurally similar mDNB (Foster et al, 1986; 1987; Williams & Foster, 1988 and see Chapter 3) and NB (Bond *et al,* 1981 and see Chapter 4), they too will have adverse effects on testicular function. Ciss *et al* (1980) reported the acute and chronic testicular toxicity of the isomers of MNT. When male and female Wistar rats were given 2 MNT (200 mg/kg/day), 3-MNT (300 mg/kg/day), or 4-MNT (400 mg/kg/day) 5 days/week for 6 months, a number of organ-, isomer- and sex-specific toxicities were observed. Testicular atrophy was observed after either 3-MNT or 4-MNT (but not 2-MNT) administration. Furthermore, all three isomers produced splenic lesions in male rats while lesions in kidney tubules were seen only after treatment with 2-MNT. In acute toxicity studies (Ciss *et al,* 1980) 2-, 3- or 4-MNT were administered orally at weekly doses of 500 or 1000 mg/kg over a period of 4 weeks. Histological examination of testicular tissue revealed that 3- or 4-MNT (but not 2-MNT) at both doses had caused severe serminiferous tubule atrophy, such that in most tubules only Sertoli cells and a few spermatogonia were present.

Considering the number of studies demonstrating the testicular toxicity of the mono-, di- and tri-nitrotoluenes there are no published reports on the effects of these compounds on testicular cell cultures. This is surprising since histological evidence from a number of studies would seem to indicate a direct effect of these compounds on the Sertoli cell, in a similar manner to that observed following the administration of other known Sertoli cell toxicants (Creasy *et al,* 1984; Foster *et al,* 1986; 1987).

With these publications and considerations in mind, the objectives of the present study were to assess the acute effects of single and multiple oral doses of the isomers of MNT *in vivo,* while Sertoli cell mono- and co-cultures were used to examine the potential of Sertoli cell and/or germ cell toxicity of either 2-, 3- or 4-MNT *in vitro.*

A dose-dependent decline in testicular weight was observed following administration of a single or multiple doses of 2-MNT, whilst only multiple dosing 4-MNT at the highest dose (750 mg/kg/dose) decreased testicular weight (Fig 5.1). In addition, fluctuations in epididymal weight following single or multiple doses of 3-MNT were also observed (Fig 5.1). These results are contrary to those of Ciss *et al* (1980) and suggest that 2-MNT may be a testicular toxicant inducing a dose-dependent decrease in testicular weight. The results obtained for testicular weight from perfused rats support this hypothesis and also suggest that 3-MNT may exert testicular toxicity, although the exact values are not comparable between experiments since perfusion fixation reduces tissue weight by up to 20% due to dehydration and shrinkage. The results from these temporal experiments suggest that both 2- and 3-MNT induce time-dependent decreases in testicular weight in common with the testicular toxicity of other compounds (e.g. mDNB and NB) although the magnitude of response was clearly less than for these other compounds 120h after treatment. Similarly epididymal weight in these perfused rats was also reduced 120h after treatment with 2- and 3-MNT.

Numerous previous studies have demonstrated that a reduction in testicular weight is usually consistent with germ cell loss and that changes in the hormonal milieu, particularly in serum FSH levels, are an indicator of major germ cell degeneration and loss. Bloch *et al* (1988) examined the reproductive toxicity of 2,4-dinitrotoluene (DNT) in the adult male rat fed DNT as 0.1 or 0.2% of the diet over a 3 week period. In addition to ultrastructural changes in Sertoli cell morphology and a reduction in epididymal weight following 0.2% DNT, circulating levels of FSH and LH were increased, the former by 25% and the latter doubling in magnitude while testosterone levels remained equivalent to controls. These authors suggested that the site of DNT action was the Sertoli cell, resulting in both inhibition of spermatogenesis and changes in the testicular-pituitary endocrine axis. Ciss *et al* (1980) did not measure serum hormone levels, however conclusions drawn from the present study (particularly that of serum FSH) following MNT administration are not consistent with a direct effect on the testis. Following single or multiple doses of 2-MNT, the levels of serum FSH only altered after a dose of 1000 mg/kg. It seems likely that this was the result of systemic toxicity rather than a direct effect of the compound on the testicular-pituitary axis. In contrast, levels of serum FSH were elevated in a dose-dependent manner following the administration of single oral doses of 3-MNT or multiple doses of 4-MNT. Based on previous investigations (de Krester & Kerr, 1983), elevations of serum FSH occur only after major germ

cell loss and major reductions in testicular weight. Using testicular weight as an index of toxicity, 2-MNT induces a dose-dependent decrease in testicular weight while 2- and 3-MNT induce time-dependent reductions in testicular and to a lesser extent epididymal weight. However, contradictory results obtained from serum FSH levels suggest that 3- and 4-MNT are testicular toxicants inducing significant increases in the levels of serum FSH. The latter results are in agreement with Ciss and co-workers (1980) who suggested that 3- and 4-MNT were testicular toxicants. In addition, marked fluctuations in the levels of serum LH and to a lesser extent testosterone were observed following the administration of single or multiple doses of all isomers of MNT. These variations however were not significantly different from controls and probably reflect the changes in serum chemistry, biochemical and general systemic toxicity of such large single and multiple oral doses of these compounds (discussed later).

The confusing indirect evidence and lack of conclusive data on testicular dysfunction following MNT administration is compounded by the unfortunate lack of testicular morphology. Although testes were perfused to observe temporal changes in testicular morphology induced by MNT, tissues were poorly fixed, such that it was impossible to accurately distinguish compound-induced disruption and artifact. The absence of individual germ cells from the complement of the normal epithelium was observed in sections taken from animals at 72 and 120h after treatment with either 2- or 3-MNT. However it was difficult to identify if this was extracellular vacuolation, the result of Sertoli cell phagocytosis or the sloughing of degenerating germ cells into the lumen, the result of poor histological fixation, since these extracellular vacuoles were observed in sections taken from control animals. Therefore, it is more likely to represent germ cells removed during histological processing, problems probably exaggerated by shrinkage artifact.

Further experiments were performed using higher single and multiple doses of either 2-, 3- or 4-MNT in an attempt to identify histological changes in testicular tissue a number of days after administration (results not shown). The LD $_{50}$'s for 2-, 3- or 4-MNT in male Wistar rats are 2100 ± 145; 2200 ± 145 and 4700 ± 330 mg/kg bodyweight respectively (Ciss *et al*, 1980). Therefore, single oral doses of 1000, 1500 and 2000 mg/kg bodyweight were administered and the perfusion technique used in previous studies employed here (see Chapter 2 and Kerr *et al*, 1984). Unfortunately most animals in the 1000mg/kg groups and all animals in the remaining groups died of overt toxicity 24h after administration. Of the remaining animals in the groups dosed with

1000 mg/kg of 2-, 3- or 4-MNT none showed any histological signs of testicular dysfunction or germ cell degeneration, while all animals in these groups showed signs of overt toxicity including cyanosis, piloerection, decreased motor activity, lack of righting reflex etc following administration. On general examination following perfusion, all had abnormal 'spotty livers', were haemopoietic and had distended darkly coloured spleens. The probable differences in toxicity to single oral doses of 1000 mg/kg and multiple doses of 750 mg/kg observed in the earlier experiments and the overt toxicity observed here is probably due to differences in the strain of rats used (Wistar derived Alpk:AP rats were used initially, while Sprague Dawley derived rats were used in the later experiments).

In addition to testicular and epididymal weights measured in earlier studies, liver, spleen and kidney weights were measured 5 days after a single or multiple oral doses of either 2-, 3- or 4-MNT. Small but significant changes in the weights of a number of these organs were observed. These included a significant reduction in liver weight after single oral doses of 3-MNT at a number of concentrations and following multiple doses of 4-MNT at all concentrations used. Reductions in spleen and kidney weight were also observed after the administration of multiple oral doses of 4-MNT. These results tend to indicate the initiation of liver, spleen and kidney toxicity which have been previously reported by other workers using multiple doses of compounds over many months (Ciss *et al*, 1980).

The dichotomy between the lack of indirect or direct evidence for testicular toxicity *in vivo* for the isomers of MNT observed in present studies and those of Ciss *et al* (1980) is probably explained by the different dosing regimes employed in each study. Since the aim of the present study was to use a suspected testicular toxicant to specifically disrupt testicular function only single oral doses were employed. Studies which report testicular degeneration and atrophy have been performed under regulatory guidelines, therefore whether they are acute or chronic studies they have one common factor; compounds are administered on a daily basis in multiple dose regimes from 5 days up to 6 months. While it is understandable that hepatic, splenic or nephrotoxicity may take place over this time period it is difficult to identify if testicular toxicity is the result of a direct effect on the testicular cells or is secondary to changes elsewhere in the body which result in testicular disruption. Although published data on the di- and tri-nitrotoluenes indicate that they may induce a direct effect on the testis, (Rickert *et al*, 1984; Levine *et al*, 1984; Lane *et al*, 1985; Bloch *et al*, 1988), further work is required to determine if the isomers of MNT have direct effects on the testis or effects secondary to whole body biochemical

changes. A more conclusive answer to the testicular toxicity of not only mono-but di- and tri- nitrotoluene may lie in studies *in vitro* involving the uses of Sertoli cells in culture in the presence or absence of germ cells similar to studies performed for the phthalates (Gray & Beamand, 1984), mDNB (Williams & Foster, 1988 and Chapter 3) and NB (Chapter 4).

Exposure of Sertoli cells in culture to 2- or 3-MNT resulted in Sertoli cell vacuolation coupled with dose-dependent germ cell exfoliation at doses in excess of 10^{-3}M and an increase in the secretion of inhibin in mono-and co-culture. Based on the present *in vitro* results as indices of toxicity 2-MNT would seem to be more toxic than 3-MNT with 4-MNT being relatively non-toxic, as effects were only observed at 5×10^{-3}M, a dose which exhibited non-specific germ cell cytotoxicity. The present results therefore suggest that 2- and 3-MNT produce specific perturbation of Sertoli cell function *in vitro*, similar to that for other Sertoli cell toxicants including mDNB (Foster *et al*, 1986; 1987), mono -2-(ethylhexyl) phthalate (Williams & Foster, 1988) and NB (Chapter 4). The present results also suggest that inhibin secretion may be another *in vitro* marker of the toxicant actions of the isomers of MNT on the Sertoli cell.

In the present study inhibin has been used as an index of Sertoli cell function *in vitro*. Following exposure of co-cultures or Sertoli cell mono-cultures to the isomers of MNT, basal inhibin secretion exhibited a reproducible alteration in response. The administration of 2-MNT to co-cultures induced significant increases in inhibin secretion at doses in excess of 10^{-4} M, reaching a peak at 10^{-3}M, while in mono-culture a significant increase was only observed at 5×10^{-3} M. In both mono-cultures and co-cultures 3-MNT at a dose of 10^{-3}M induced significant increases in inhibin secretion while in contrast 4 MNT at a dose of 5×10^{-3}M induced significant decreases in inhibin secretion in both mono- and co-culture. The cause of these changes in inhibin secretion following toxicant administration is unknown, however they were observed repeatedly in both mono- and co-culture; it is therefore unlikely to be artifactual or due to the exfoliation of selective germ cell type(s), a possibility that arises because of the probable control of inhibin secretion by selective germ cell types (Pineau *et al*, 1990). The consistently higher levels of inhibin secretion by Sertoli cell-germ cell co-cultures in the present study when compared with that of Sertoli cell mono-cultures was similar to that observed in other studies (Chapter 3 and 4) and is further evidence for germ cell modulation of Sertoli cell inhibin secretion.

Unfortunately the levels of inhibin in peripheral blood collected in the *in vivo* experiments were not measured and testicular interstitial fluid (IF)

was not collected from the animals. It is therefore impossible to make direct *in vivo - in vitro* comparisons for inhibin secretion from these animals. An increase in the testicular IF levels of inhibin in treated animals following either 2-, 3- or 4-MNT administration may well have indicated Sertoli cell pertubation *in vivo* - (similar to that observed after NB- and MAA- administration - see Chapters 4 and 6 respectively). Unfortunately at the time of the experiment samples of peripheral serum and not plasma were collected from MNT treated rats. Subsequently, it has been demonstrated that the clotting of blood during serum formation results in the release of unknown factors which cross-react or interfere in the inhibin RIA (see Chapter 2 and Maddocks & Sharpe, unpublished data). Further work is required in order to correlate possible changes in inhibin secretion observed *in vitro* with those measured in testicular IF and peripheral plasma *in vivo*.

Indirect evidence for changes in the pattern of secretion of inhibin *in vivo* may be reflected in the alterations in serum FSH levels following MNT administration. Historically, FSH has been postulated to positively regulate inhibin secretion whilst inhibin has been presumed to negatively regulate FSH. Therefore changes in the secretion of FSH are presumed to reflect the opposite change in the levels of inhibin. Following the administration of 2-MNT *in vivo*, a dose-dependent decrease in serum FSH levels was observed, and in contrast, a dose-dependent increase in serum FSH levels was observed following the administration of 3-MNT. The latter could be explained by the increase in inhibin secretion observed in culture, however this increase was also observed *in vitro* following the administration of 2-MNT which, *in vivo*, resulted in a decrease in serum FSH levels. The probable differences in response *in vitro* may lie in the lack of testicular toxicity observed *in vivo* following a single oral dose of either 2-, 3- or 4-MNT in comparison to multiple oral doses of these compounds usually administered over many months. It is, however, possible that metabolite(s) of MNT are responsible for the effect observed *in vivo*. Possibly the build-up of the parent compound or metabolite(s) may be required to induce testicular disruption *in vivo*, in which case continual daily administration of the compounds would allow the gradual build-up of the parent compound or toxic metabolite(s) within the testis such that testicular degeneration would eventually become apparent. Unfortunately using these dose regimes it is impossible to identify exactly when the first signs of testicular toxicity begin to occur or in which particular testicular cells.

As yet it has not been determined if 2-, 3- or 4-MNT are directly responsible for the testicular atrophy observed by Ciss *et al* (1980), or are the

result of indirect secondary changes or the build-up of toxic metabolites of 2-, 3- or 4-MNT. Indeed, a number of the urinary metabolites of 3- and 4-MNT in rats following a single oral dose differ only quantitatively while those of 2-MNT also differ qualitatively (Chism *et al*, 1984). Studies on the metabolism and excretion of 2-, 3- or 4-MNT in Fischer 344 rats have shown that these compounds are rapidly excreted (86, 68 and 74% of an oral dose of 2-, 3- or 4-MNT respectively excreted within 72h) (Chism *et al*, 1984). Metabolism studies in rats have demonstrated that following a single oral dose of any of the MNT's, all metabolites are excreted into the urine; most of these originate from the nitrobenzyl alcohols. The most abundant excreted metabolites of 2-MNT were S-(2-nitrobenzyl) N-acetylcysteine (12%), 2-nitrobenzyl glucuronide (14%) and a metabolite tentatively identified as a sulphur containing conjugate of 2-amino-toluene (16%; conjugated through the benzyl group). Those of 3-MNT were 3-acetamidobenzoic acid (12%), 3-nitrobenzoic acid (21%) and 3-nitrohippuric acid (24%). The major urinary metabolites of 4-MNT were 4-nitrohippuric acid (13%), 4-acetoamidobenzoic acid (27%) and 4-nitrobenzoic acid (28%) (Rickert, 1987). Therefore it is possible that testicular toxicity induced by any of the isomers of MNT may not be a direct result of the parent compound but a metabolite(s), possibly produced by other organs (e.g. the cytochrome P_{450} system of the liver) or the testis directly (e.g. the Sertoli cell - similar to that described for mDNB by Foster, 1989). Since 3- and 4-MNT have been reported to be testicular toxicants (Ciss *et al*, 1980) it is tempting to speculate that a metabolite of these two compounds is the possible testicular toxin which, through multiple dosing, builds-up in concentration to such an extent that it disrupts testicular function. If this is the case then further work is required using the known metabolites of 3- and 4-MNT to identify their testicular toxicity both *in vivo* and *in vitro*.

Calculations based on the pharmacokinetics of the elimination of the isomers of MNT suggests that, after a single oral dose of MNT at a dose of 250 mg/kg, peak blood levels (and therefore intratesticular levels) would be approximately 10^{-6}M, whilst at a dose of 1000 mg/kg they would be approximately 10^{-3}M (Rickert *et al*, 1981; 1987). Based on these results, doses of compound shown to affect the Sertoli cell and its secretions *in vitro* may not be exactly representative of a single oral dose of compounds when administered *in vivo*. These calculations however are based on a number of assumptions and therefore may be inaccurate. It is, however, possible that absorption, distribution and elimination characteristics of the parent compounds *in vivo* do not allow a concentration to accumulate within the testis sufficient to cause

testicular toxicity and consequently the results obtained *in vitro* may not represent a physiological situation. Furthermore if MNT is metabolised by an organ such as the liver and it is this metabolite(s) which is responsible for the testicular disruption then further work is required *in vitro* using known metabolites of MNT to assess their effects on the Sertoli cell *in vitro* at concentrations relevant to those produced *in vivo* following MNT administration.

In conclusion, for the purposes of this study, the isomers of MNT could not be used to induce specific testicular disruption in the context of using them to investigate the process of spermatogenesis.

CHAPTER 6.

METHOXYACETIC ACID.

6.1. INTRODUCTION.

The glycol ethers are a family of solvents often referred to as "Cellosolves" which are an important class of industrial solvents and emulsifiers, due in large part to their miscibility with both water and many organic solvents (National Institute of Occupational Safety and Health-N.I.O.S.H., 1983). N.I.O.S.H. estimates that each year betweeen 200,000 and 2,000,000 workers are exposed to each of six glycols, glycol ethers or glycol ether acetates (reviewed by Hardin, 1983). Consumer applications of these ether and ether acetates include surface coatings (paints, laquers, varnishes etc), fingernail polishes and polish removers, liquid cleaning products, degreasing agents and spotting fluids, hydraulic brake fluids, printing and writing inks and textile and leather dying solutions.

Some of the economically important glycol ethers in the chemical industry are the low molecular weight ethylene glycol mono-n-alkyl ethers, examples of which are ethylene glycol monomethyl ether (EGME), ethylene glycol monoethyl ether (EGEE) and ethylene glycol monobutyl ether (EGBE). These compounds are also known under their synonyms of 2-methoxyethanol, 2-ethoxyethanol and 2-butoxyethanol respectively. EGME, because of its unique solvent characteristics, has found numerous applications as a component in surface coatings, inks, dyes, hydraulic brake fluid and water-based cleaners.

Due to their low vapour pressure and high rate of dermal absorption the glycol ethers are readily absorbed either percutaneously, or by inhalation or ingestion. Sufficient exposure can occur through contact with skin to produce toxicity in animals and humans (Rowe & Wolfe, 1982). In experimental animals the most prominent and well-recognised targets of glycol and glycol ether toxicity are the central nervous, renal and haemopoetic systems (Gosselin *et al*, 1976), whilst reproductive system involvement is also noted (Miller *et al*, 1981). Fatty degeneration of the liver and pulmonary oedema have also been reported (Rowe *et al*, 1982). High doses may cause death either by respiratory tract arrest or renal failure. Severe haematological and central nervous system disturbances have also been reported following occupational exposure to EGME (Donley *et al*, 1936; Ohi *et al*, 1978). The latter authors reported that exposure was almost exclusively cutaneous, vividly demonstrating that these compounds readily penetrate the skin in toxic amounts.

The possible reproductive toxicology of chemicals such as the glycol ethers is a matter of growing public concern. Recently, attention has been focussed on the effects of glycol ethers on the male reproductive system, on foetal and embryonic development, and on the haemopoietic system. It has been

known since the late 1930's that some of these glycol ethers can produce testicular damage in animals. In 1936 Wiley *et al* reported testicular disruption induced by inhalation of EGME in rats and mice. Two years later the same group reported equivalent effects in rabbits following EGME injection. Later studies, however, were contradictory. Morris *et al* (1942) showed that in rabbits and rats no effects were observed. Because of this inconsistency the potential of glycol ethers to induce testicular toxicity was not widely appreciated until much later. More recent work by Nagano *et al*, (1979) on the testicular effects of a range of ethylene glycol mono-n-alkyl ethers in mice served to re-awaken interest in these compounds. These authors found that EGME, EGME acetate and EGEE were the most potent in their effects on the testes. The earliest effects on relative testicular weight with EGME occurred at a dose level of 250 mg/kg/d after 5 wks of oral administration. Thus they determined a ranking order for the production of testicular damage in these species to be EGME (and its acetates)> EGEE (and its acetates)> EGBE. The inconsistencies of previous investigators may be attributed in part to impurities in the solvents used in their studies. Since then the glycol ethers have been shown to be testicular toxicants in a number of species including the rat, mouse, rabbit, dog and chicken, a species variation wider than most testicular toxicants (Hardin, 1983). Exposure to EGME for 2 weeks at a dose of 1000 ppm in the rat, mouse and rabbit produced a decrease in testicular weight as well as adverse effects on the bone marrow and lymphoid tissue in rats, mice and rabbits (Miller *et al*, 1983). The changes in the seminiferous epithelium of rats after 9 days of inhalation exposure to 1000 ppm EGME were diffuse and severe with degeneration and necrosis of the seminiferous epithelium in addition to the formation of spermatid giant bodies.

Up to this point in time, the reproductive lesions found in male animals treated with glycol ethers were generally described as "testicular or tubule atrophy", and only recently has more detailed information on the development of the lesion formed by both EGME and EGEE after oral administration to rats been published (Foster *et al*, 1983). These authors reported that 24h after a single oral dose of EGME to rats at a concentration of 100 mg/kg, the initial testicular lesion appeared to be a depletion of primary spermatocytes. In addition, 16h after a single oral dose of 500 mg/kg, spermatocyte mitochondrial swelling and disruption, cytoplasmic vacuolation and early condensation of nuclear chromatin were observed (Foster *et al*, 1983). EGME has also been found to affect primary and secondary spermatocytes within 24h of exposure to 1000 ppm, with some vacuolation of Sertoli cells also

occurring (Samuels, 1983). Doe *et al* (1983) also observed degeneration of primary spermatocytes and spermatids, with spermatogonia, Sertoli and Leydig cells apparently unaffected in rats exposed to 300 ppm EGME for 10 days. In rats exposed to 150 mg/kg EGME for 4 days, spermatocytes and round spermatids were necrotic and often missing (Chapin *et al*, 1984).

In rats killed at various times after exposure to EGME (Chapin *et al*, 1984) or EGEE (Creasy *et al*, 1984), the meiotic spermatocytes in the pachytene phase of development were found to be the most susceptable to these toxicants, whereas cells in the earlier (leptotene/zygotene) and later (early spermatid) phases were damaged only after exposure to high doses or for a long time period. Further studies by Chapin and co-workers (1985) described the initial morphological changes observed in the reproductive system of male rats following EGME treatment, to observe the morphogenesis of the lesion and to correlate histological changes with ABP activity found in fluid collected at the rete testis after efferent duct ligation. Using Fischer-344 rats gavaged with 150 mg/kg/d EGME for 5 days/wk they detected necrotic changes in some meiotic and premeiotic spermatocytes 24h after a single dose. These effects were magnified following subsequent doses with more necrotic pachytene and meiotic spermatocytes than necrotic stage I pachytene spermatocytes, suggesting a selectivity of toxicity at various spermatogenic stages. Pronounced maturation depletion occurred at later time intervals and was evident as an absence of round spermatids from tubules in stages I and III. These effects continued to occur until by 7 to 10 days post-treatment only Sertoli cells, spermatogonia and late stage spermatids populated the epithelium. No treatment-related changes in the relative amounts of ABP were detected. These experiments suggested that the degree of testicular lesion induced by EGME was dependent upon the dose and frequency of administration, lower single doses affecting a few specific germ cells (pachytene spermatocytes) at particular stages of the spermatogenic cycle whilst higher single and multiple doses affected a larger population of pachytene spermatocytes within a number of stages.

Further work established that these glycol ethers were not the instigators of damage, but specific metabolites formed *in vivo* from the parent compounds were the testicular toxicants. The glycol ethers that are alkoxyethanols are converted primarily to the corresponding alkoxyacetic acids, which in some cases are conjugated with glycine (Hutson *et al*, 1979; Jonsson *et al*, 1978; 1982; Miller *et al*, 1983; Cheever *et al*, 1984; Moss *et al*, 1985). This conversion is thought to be mediated by alcohol dehydrogenase, presumably via the alkoxyacetaldehyde intermediate (Jonsson *et al*, 1982; Miller *et al*, 1983;

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Moss *et al*, 1985) resulting in the formation of methoxyacetic acid (MAA) and ethoxyacetic acid (EAA) from EGME and EGEE metabolism respectively. MAA (Foster *et al*, 1983; Miller *et al*, 1982) and EAA (Foster *et al*, 1983; 1984) produce the same degree of toxicity as the parent compound, and methoxyaldehyde, the postulated intermediate in the conversion of EGME to MAA, causes similar effects (Foster *et al*, 1985). Pyrazole, an alcohol dehydrogenase inhibitor, blocks the conversion of EGME to MAA and protects against toxicity (Moss *et al*, 1985). Alternatively, propylene glycol monobutyl ether (PGME), another in the class of ethers, has none of the toxic effects of EGME, although it differs from EGME only by a single methyl group (Miller *et al*, 1981; Doe *et al*, 1983; Samuels *et al*, 1984). Unlike the alkoxyethanols, which are primary alcohols, PGME is a secondary alcohol and therefore a poor substrate for alcohol dehydrogenase (Von Wartburg *et al*, 1964). It is extensively metabolised to CO_2. Metabolism of dipropylene glycol monomethyl ether (DPGME)-(Landry *et al*, 1984), which is also not a testicular toxicant, is similar to that of PGME (Miller *et al*, 1985). Thus MAA has been identified as the major urinary metabolite following oral EGME administration to rats (Miller *et al*, 1983), whilst EAA and its glycine conjugates are known metabolites of EGEE, and butoxyacetic acid (BAA) that of EGBE. Miller *et al* (1982) demonstrated that the toxic properties of MAA are remarkably similar to EGME and concluded that the effects of EGME are probably due to its metabolite-MAA.

The administration of MAA to rats has been reported to cause selective and stage-specific destruction of pachytene spermatocytes. These effects are dose-dependent and at high doses (650 mg/kg) result in pachytene spermatocyte degeneration at all stages of the spermatogenic cycle other than early- to mid- stage VII (Foster *et al*, 1983; 1984; 1987; Creasy *et al*, 1985). Why these particular subgroups of pachytene spermatocytes are resistant to MAA remains a mystery. These findings were supported by studies in which male rats were mated at various times after exposure to EGME (Oudiz *et al*, 1984; Mc Gregor *et al*, 1984; Chapin *et al*, 1985). Recovery studies suggest that a high dose of EGME may cause irreversible damage (Foster *et al*, 1983; Rao *et al*, 1983; Oudiz *et al*, 1984).

EAA has also been shown to produce similar gross testicular damage to that seen with the parent glycol ether, after repeated, high dose, oral administration (Foster *et al*, 1983; Creasy *et al*, 1984). Gray *et al* (1985) reported that repeated administration of high doses (868 mg/kg/d for 4 days) of BAA to immature (31 day old) rats had no effect on testicular weight or testicular histology. However Foster *et al* (1987) demonstrated the effects of MAA, EAA

and BAA on early testicular events *in vivo* following single oral administration to mature rats and used testicular Sertoli cell cultures to re-evaluate their findings in the *in vitro* situation. They demonstrated *in vivo* that pachytene and dividing spermatocytes in stages XIII, XIV and I were the initial, primary site of toxicity for MAA and EAA, with degeneration of these cells occurring within 24h of treatment at all doses of MAA and at the highest dose of EAA, whilst BAA had no discernable effect on the testis at equimolar doses. The development of testicular lesions was basically similar to that described for the corresponding glycol ethers (Creasy & Foster, 1984). Addition of MAA or EAA to testicular cell cultures at concentrations equivalent to the steady state plasma levels of MAA produced specific loss of pachytene spermatocytes. MAA was more potent than EAA. They therefore concluded that; a) spermatogenesis appeared to be sensitive to MAA exposure; b) the production of testicular toxicity by alkoxyacetic acids diminished with increasing chain length; c) a good correlation between the effects of these compounds *in vivo* and *in vitro* existed.

The mechanism by which MAA-induced pachytene spermatocyte degeneration was investigated further *in vitro*. Exposure of Sertoli cells in culture to MAA, but not EGME itself, decreased the production of lactate but had no effect on protein synthesis or cell viability (Beattie *et al*, 1984). Exposure to EGME decreased testicular lactate levels *in vivo* (Beattie *et al*, 1985). These observations are significant since pachytene spermatocytes cannot utilise glucose and are dependent on lactate provided by Sertoli cells for an energy source (Jutte *et al*, 1982). Therefore it was suggested that an inhibition of lactate production *in vitro* and possibly *in vivo* resulted in pachytene spermatocyte degeneration. Furthermore Williams and Foster (1988) have demonstrated in culture a differential stimulation of lactate, (and not pyruvate) by toxicants. In particular mono-2-(ethylhexyl) phthalate partially inhibited hormonally stimulated pyruvate but not lactate secretion by Sertoli cells in culture, probably through an inhibition of lactate dehydrogenase (LDH-C4) and thus removed lactate as an energy source for germ cells, particularly pachytene spermatocytes. However Gray *et al* (1985) demonstrated that exogenous administration of lactate to mixed Sertoli-germ cell co-cultures did not protect against MAA-induced pachytene spermatocyte degeneration suggesting that a lack of endogenously produced lactate was not a prerequisite for pachytene spermatocyte necrosis.

Bartlett *et al* (1988) utilised the selective effects of MAA *in vivo* to study the effect of pachytene spermatocyte degeneration on the pituitary-testcular endocrine axis. A single oral dose of MAA (650 mg/kg) was administered and hormonal measurements made in addition to histological

observations made at specific time points up to 70 days post-treatment, (the time for at least 5 spermatogenic cycle in the rat). The selection of this dose of MAA was based on previous results demonstrating that it selectively removed most pachytene spermatocytes. Results indicated that following the depletion of pachytene spermatocytes, Sertoli cell function was altered. Serum follicle stimulating hormone (FSH) and interstitial fluid ABP levels increased biphasically at 3 and 21 days post-treatment but not in the intervening period. Testosterone and luteinising hormone (LH) levels remained comparable to controls. The two periods of increased FSH and ABP coincided with the times of greatest decrease in testicular weight. These data suggest that endocrine/paracrine function of the Sertoli cells was affected post-treatment possibly as a direct result of pachytene spermatocyte depletion.

Recently Ratnasooriya & Sharpe (1989) have used fertility tests on male rats following MAA administration to detect if other germ cells were affected by MAA in a non-lethal manner, or alternatively that due to a lack of paracrine interaction between pachytene spermatocytes and Sertoli cells an impairment of Sertoli cell function may have occurred and therefore affected subsequent germ cell development. Previous investigators have demonstrated that the administration of MAA or EGME to rats causes small changes in the duration of the spermatogenic cycle (Chapin *et al*, 1984; Creasy *et al*, 1985). Ratnasooriya and Sharpe (1989) found that apart from the specific loss of pachytene and later spermatocytes at all stages other than early to mid stage VII, other changes occurred at 21 days post-treatment. These included a reduction in the number of pachytene spermatocytes at late stage VII, a retention of sperm at stages IX-XIV and an increased degeneration of pachytene spermatocytes and round spermatids at stage VII and of secondary spermatocytes at stages XIV-I. They concluded that whilst these deleterious changes in spermatogenesis may occur secondarily following MAA treatment, for the most part spermatogenesis proceeds normally and fertility is largely maintained despite a massive but transient decrease in sperm output corresponding to previously degenerated pachytene spermatocytes.

In conclusion, both *in vivo* and *in vitro* data suggests that an initial effect on pachytene spermatocytes takes place, and that the mode of action of MAA/EGME in producing testicular cell degeneration based on *in vitro* data suggests that the enzyme LDH-C4 may be inhibited so reducing the production of lactate, a necessary energy source for germ cells, particularly pachytene spermatocytes. In addition, there is a spermatogenic stage-related susceptability to MAA in the rat, in which the degree of pachytene spermatocyte degeneration

is directly proportional to the dose administered, which may therefore be utilised in studying stage-related differences in normal function *in vivo* due to the effects of MAA being germ cell specific, stage-specific and dose-related.

MAA may prove to be extremely useful in the selective intervention of spermatogenesis *in vivo* and the study of events post-administration, and, when extrapolated to the normal situation, may aid in our understanding of spermatogenesis. The effects of MAA are limited by dose and its cytotoxicity is specifically directed towards pachytene spermatocytes at specific stages of spermatogenesis. To this end it may be useful *in vivo* to remove these cells and to study temporal changes that occur in both the histological and hormonal aspects of testicular function and possible paracrine interactions which occur between pachytene spermatocytes and Sertoli cells both *in vivo* and *in vitro* .

6.2. METHODS.

6.2.1. Methods for assessment of the effects of MAA *in vivo* .

6.2.2. Reagents.

MAA was obtained as a liquid from Aldrich Chemical Co. (Gillingham, Dorset, U.K). Analysis by gas chromatography/mass spectrometry for composition before use found it to be >99% pure. The vehicle for MAA administration was 0.9% saline. Due to the acidic nature of MAA (approximate pH 0.5) dosing solutions were correct to pH 7.4 before administration using concentrated sodium hydroxide.

6.2.3. Animals.

Rats were of the Sprague Dawley derived strain bred in the Centre for Reproductive Biology in Edinburgh, U.K.

6.2.4. Experimental Protocol.

Adult male rats (80-130 days old) were allocated randomly 8/cage and maintained under normal animal house conditions: (12h:12h light dark cycle, 21^0C and 35-60% humidity throughout the course of the experiment). Food and water were available *ad libitum*.

At the beginning of the experiment (time 0) rats were administered MAA by oral gavage at a single dose of 650 mg/kg bodyweight (or 225 mg/ml of saline - pH 7.4). Control groups were administered vehicle alone (2.9

mls/kg). Animals were weighed daily for the first 14 days post-treatment. Significant decreases in body weight were considered to indicate overt toxicity. At selected time intervals (1, 3, 7, 14, 21, 28, 35 or 42 days post-treatment) rats were subjected to one of the following procedures;

a) 6 rats/group were killed by CO_2 inhalation followed by cervical dislocation, the testes were removed and weighed, blood was collected immediately after death for hormone measurement (see section 2.3.) and testicular interstitial fluid (IF) was drip collected overnight (see section 2.2.1.) also for hormone measurements. Serum hormones measured were FSH, LH and testosterone and hormones measured in IF were testosterone and inhibin.

b) 2 rats/group were killed by CO_2 inhalation followed by cervical dislocation, both testes were dissected from the animal, blotted dry and weighed. In one testis, a small incision was made at the caudal end of the testicular capsule. This testis was placed into M199 containing 20mM Hepes and kept in ice for isolation of seminiferous tubules (see section 2.6.1.). The contralateral testis was placed into a polystyrene tube for testicular IF collection at 4°C. Blood and testicular IF collected from these animals was stored at -20°C for hormone measurement (see above).

6.2.5. Methods for the assessment of MAA *in vitro.*

6.2.5.1. Preparation of seminiferous tubule cultures.

Seminiferous tubules were isolated from testes removed from MAA- or vehicle-treated rats, (under the conditions described in section 2.6.) and cultured in the absence or presence of MAA and/or rat FSH and/or dibutyryl cyclicAMP, such that four different combinations of MAA treatment at each time point were used. These were:

a) seminiferous tubules isolated from animals treated with MAA.
b) seminiferous tubules isolated from animals treated with MAA and cultured in the presence of 10^{-4} M MAA.
c) seminiferous tubules isolated from animals treated with vehicle alone.
d) seminiferous tubules isolated from animals treated with vehicle alone and cultured in the presence of 10^{-4} M MAA.

Aliquots of isolated 10cms of seminiferous tubules from each of these groups were cultured in the absence or presence of rat FSH or dibutyryl cyclicAMP. The concentration of MAA in culture was 15- fold less than peak blood levels evident after the administration of MAA at 650 mg/kg (Foster - personal communication). Therefore, this concentration in culture was approximate to or less than an appropriate physiological level observed *in vivo*.

6.2.5.2. Treatment of seminiferous tubules in culture.

MAA was administered to isolated seminiferous tubule cultures (groups b and d) at a final concentration of 10^{-4} M in saline (0.3% v/v) corrected to pH 7.4. Seminiferous tubules cultured in the absence of MAA (groups a and c) received an equal volume of vehicle.

In each of the treatment groups a to d seminiferous tubules were cultured in medium alone ($\pm$ MAA) or in the presence of dibutyryl cyclicAMP (at a final concentration of 2.5 µg/well) or NIAMDD-rat FSH-S-11 (at a final concentration of 0.1ng/well). Both dibutyryl cyclicAMP and rat FSH were applied directly to the cultures in culture medium at the time of toxicant (or vehicle) administration.

Culture medium was removed by aspiration and replaced every 24h with the appropriate treatment ($\pm$ MAA $\pm$ stimulation) over a culture period of 72h. Medium was stored at -20°C and assayed for inhibin content.

Treatments caused no appreciable alteration of culture medium pH. All experiments were performed with a minimum of four replicates for each treatment group.

6.3. RESULTS.

6.3.1. Effect of MAA on testicular weight.

MAA induced a time-dependent decrease in testicular weight up to 14 days post-treatment (Fig 6.1). Between 14 and 35 days post-treatment a gradual but progressive recovery in testicular weight in MAA-treated rats was observed in comparison to respective vehicle-treated groups. At 35 and 42 days post-treatment testicular weight in MAA-treated rats was not significantly different when compared with respective saline-treated controls.

Fig 6.1. Effect of a single oral dose of 2-methoxyacetic acid (650 mg/kg bodyweight) (■) or vehicle alone (2.9 mls/kg) (□) on testicular weight in groups of rats at intervals after dosing. Values are means ± SD for 8 rats (*P<0.01, in comparison to respective controls).

6.3.2. Effect of MAA on the serum levels of FSH.

Within 24h of a single oral dose of MAA serum FSH levels were elevated significantly and remained higher than respective controls 3 days post-treatment (Fig 6.2). Between 3 and 7 days post-treatment, serum FSH levels in MAA-treated rats decreased to back within the control range and remained at this level up to 14 days post-treatment. A second significant rise in serum FSH levels then occurred at 21, 28 and 35 days post-treatment but by 42 days after MAA administration serum FSH levels had again declined to within the control range (Fig 6.2).

6.3.3. Effect of MAA on the serum levels of LH.

Significant increases in serum LH levels in MAA-treated rats were observed at 21 and 28 days post-treatment (Fig 6.2), the highest levels being measured at 21 days post-treatment, with a subsequent gradual decline between 28 and 42 days post-treatment. Levels were not significantly different from respective controls at 35 and 42 days post-treatment.

6.3.4. Effect of MAA on interstitial fluid (IF) levels of inhibin.

IF levels of inhibin were increased significantly at 1 and 3 days post-MAA treatment to an equivalent extent, but by 7 days, and up to 42 days, inhibin levels in IF had returned to control values (Fig 6.3).

6.3.5. Effect of MAA on the serum and IF levels of testosterone.

Serum and IF levels of testosterone showed marked between-animal variation in both control and MAA-treated groups and, overall, there was no evidence for any significant change as a result of treatment (Fig 6.4).

6.3.6. Effect of MAA administration *in vivo* and/or *in vitro* on basal and stimulated inhibin secretion by isolated seminiferous tubules

When compared with equivalent controls, major changes occurred in the secretion of inhibin by cultured seminiferous tubules isolated from rats treated *in vivo* with MAA (Figs 6.6 to 6.11). Significant differences were also evident in inhibin secretion in cultures derived from seminiferous tubules isolated from MAA- and saline-treated rats cultured in the presence or absence of 10^{-4} M MAA (Figs 6.12 to 6.17). However, the pattern of inhibin secretion over the 3 day culture period between seminiferous tubules isolated from MAA-treated animals and cultured in the absence (see methods - group a) or presence

Fig 6.2. Effect of a single oral dose of 2-methoxyacetic acid (650 mg/kg bodyweight) (■) or vehicle alone (2.9 ml/kg) (□) on serum levels of FSH (a) or LH (b) in groups of rats at intervals after dosing. Values are means ± SD for 8 rats (*P<0.01, **P<0.001, in comparison to respective controls).
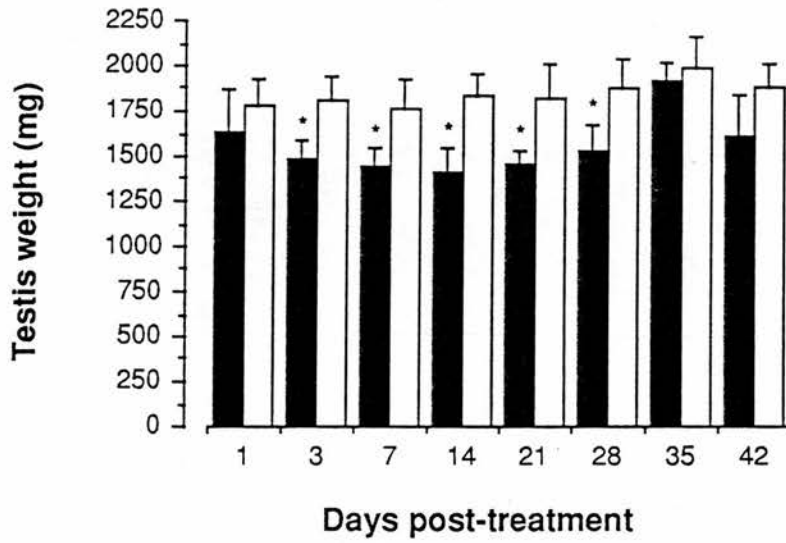
Fig 6.3. Effect of a single oral dose of 2-methoxyacetic acid (650 mg/kg bodyweight) (■) or vehicle alone (2.9 mls/kg) (□) on immunoactive inhibin levels in testicular interstitial fluid (IF) from groups of rats at intervals after dosing. Values are means ± SD for 8 rats (**P<0.001, in comparison to respective controls).
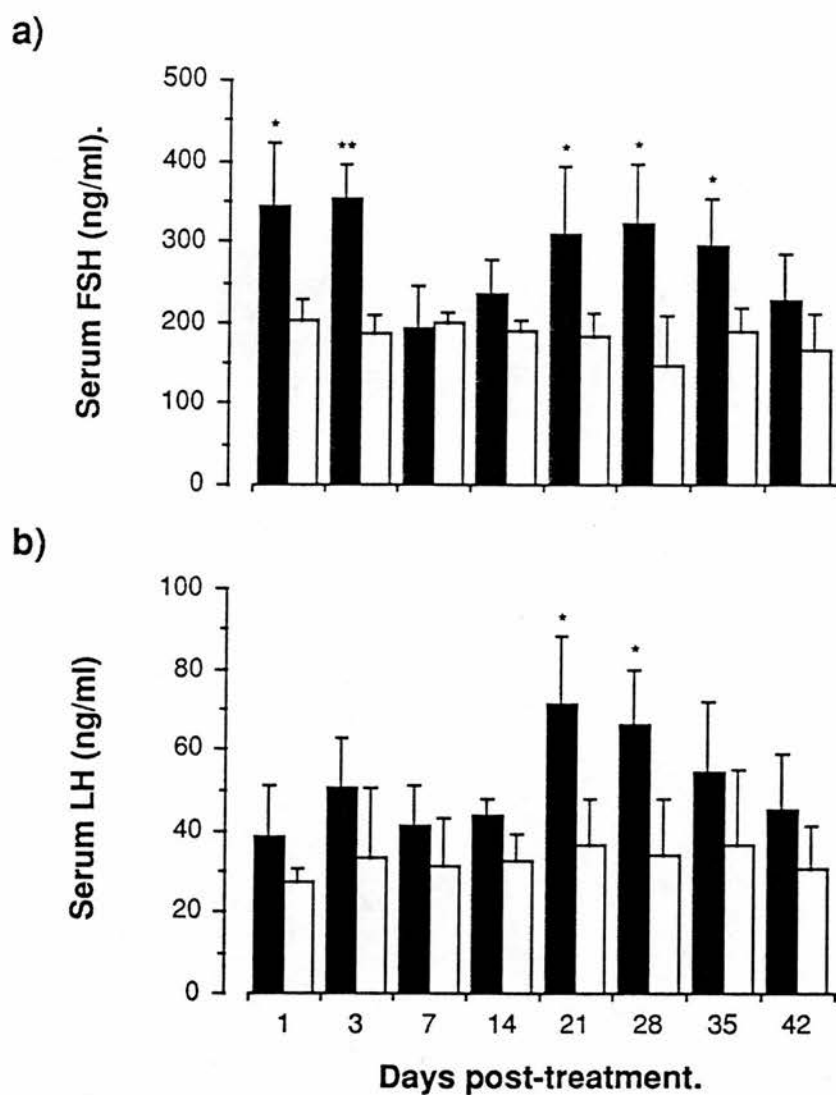
a)

b)
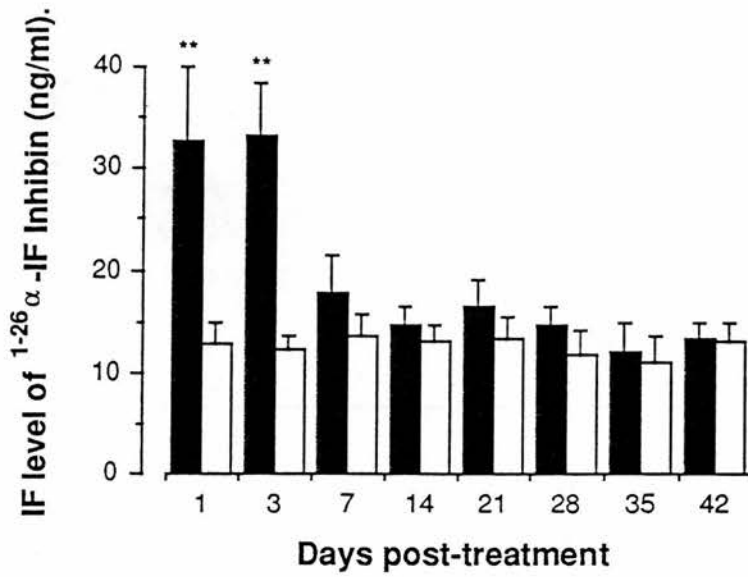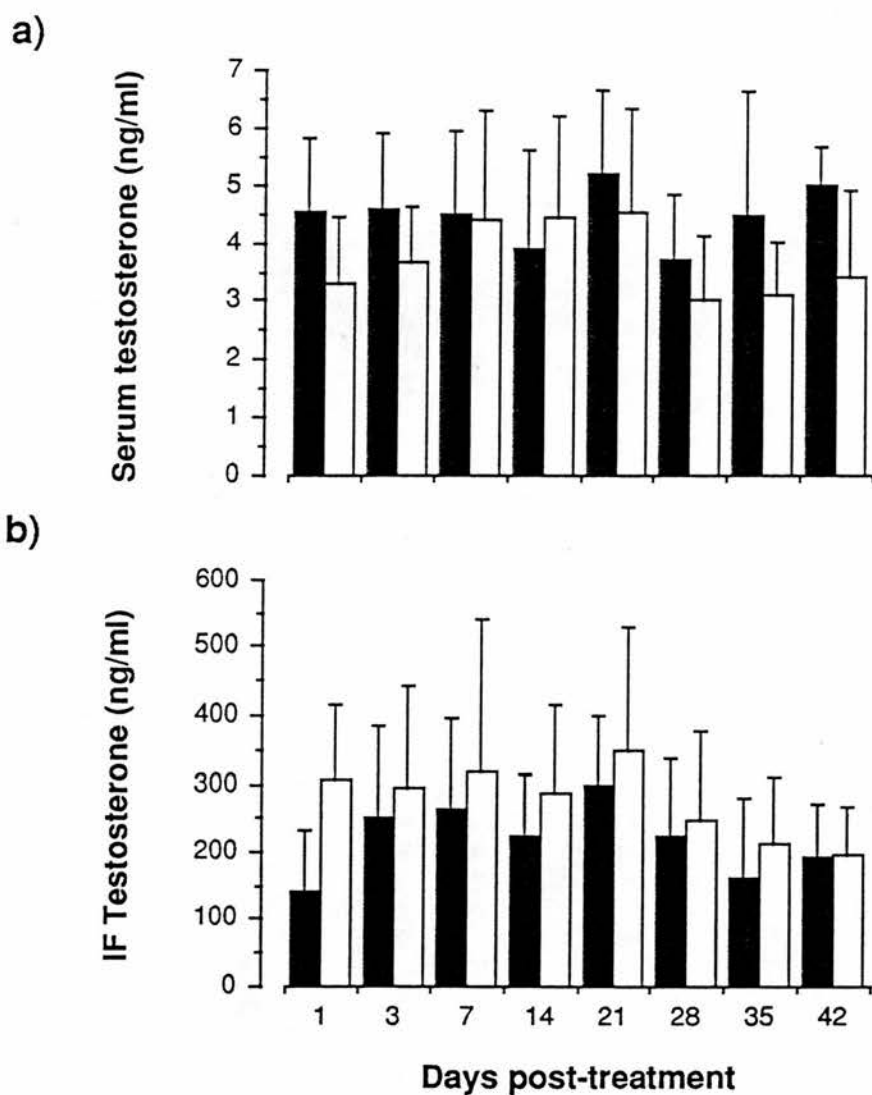

Fig 6.4. Effect of a single oral dose of 2-methoxyacetic acid (650 mg/Kg bodyweight) (■) or vehicle alone (2.9 mls/kg) (☐) on testosterone levels in serum (a) and testicular interstitial fluid (IF) (b) from groups of rats at intervals after dosing. Values are means ± SD for 8 rats. No significant differences (P>0.05) were observed.

(see methods - group b) of 10^{-4} M MAA did not differ significantly from one another. Therefore results for group b i.e. seminiferous tubules isolated from animals treated with MAA and cultured in the presence of 10^{-4} M MAA have been omitted for the sake of clarity.

In all experiments using seminiferous tubules isolated from MAA-treated or saline-treated rats and cultured in the presence or absence of MAA over a 3 day period, dibutyryl cyclicAMP and rat FSH induced 3- to 5-fold increases ($P<0.01$) in inhibin secretion in comparison to respective basal levels (see Figs 6.6 to 6.17).

6.3.7. Inhibin secretion by seminiferous tubules isolated at successive time intervals after MAA-treatment *in vivo*.

The effect of a single oral dose of MAA on inhibin secretion by seminiferous tubules isolated at successive time intervals after MAA are shown together with data for saline-treated controls after 24, 48 and 72h in culture in Figs 6.6, 6.7 and 6.8 respectively. Results for a duplicated experiment using seminiferous tubules isolated from different animals are shown in Figs 6.9, 6.10 and 6.11 respectively.

Under both basal and stimulated conditions in culture and at 24, 48 and 72h after isolation changes in the pattern of inhibin secretion were observed. These were similar in both the presence and absence of stimulation and consistent over the culture period (see Figs 6.6, 6.7 and 6.8 respectively). These changes coincided with the generation and progression of germ cell-specific lesions induced by MAA post-treatment. Based on previous investigations (Bartlett *et al*, 1988; Ratnasooriya & Sharpe, 1989) MAA, at the dose administered in this study, causes major or complete (80-100%) loss of pachytene and later spermatocytes at all stages other than early- to mid-stage VII of the spermatogenic cycle between 1 and 3 days post-treatment. These histological changes are well characterised (Bartlett *et al*, 1988) while subsequent effects on spermatogenesis have also been identified through changes in; sperm output and fertility (Chapin *et al*, 1986; Ratnasooriya & Sharpe, 1989), testicular histology and changes in the hormonal milieu post-treatment (Bartlett *et al*, 1988). Specifically, Bartlett and co-workers (1988) have demonstrated that the effects of MAA are subsequently manifest as depletion/loss of mainly round (days 7-14) and elongate (days 21-28) spermatids as a result of maturation depletion. Based on previous data (Bartlett *et al*, 1988; Ratnasooriya & Sharpe, 1989) schematic representations approximating the type and degree of germ cells either degenerating (at 1-3 days)

or absent at later time intervals after MAA treatment are shown in Fig 6.5. In addition, in order to evaluate a possible relationship between Sertoli cell inhibin secretion and its modulation by the absence or presence of specific germ cell types (as suggested by Pineau *et al*, 1990), further schematic representations of the progression and degree of the MAA-induced lesion are illustrated where appropriate (i.e. see Fig 6.6 for an example).

Seminiferous tubules isolated from animals at 1 and 3 days after MAA treatment showed a significant elevation in basal secretion of inhibin irrespective of whether measurements were made after 24, 48 or 72h in culture (Figs 6.6, 6.7 and 6.8 respectively). Basal inhibin levels in culture medium conditioned by seminiferous tubules isolated from MAA-treated rats at 7 and 14 days post-exposure were not significantly different from respective controls at all culture time intervals (Figs 6.6, 6.7 and 6.8). In contrast to the increase in basal inhibin secretion observed at 1 and 3 days post-treatment the secretion of inhibin from tubules isolated at 21 and 28 days post-MAA treatment were drastically reduced in comparison to respective control cultures (Fig 6.6). This effect was consistent over the time of culture being identified at 24, 48 and 72h post-isolation (Figs 6.6, 6.7 and 6.8 respectively); indeed at the latter two time intervals basal inhibin levels were close to the minimum detection limit of the assay. At the remaining time intervals after MAA-treatment basal inhibin secretion by seminiferous tubules isolated from MAA-treated rats was not significantly different from basal control levels.

The pattern of stimulated inhibin secretion in seminiferous tubule cultures was in most instances similar to that observed under basal conditions, however the exact values for inhibin secretion in both dibutyryl cyclicAMP- and rat FSH-stimulated cultures were significantly greater than basal values at most points. Inhibin levels in culture were significantly increased by dibutyryl cyclicAMP at 1 day and by rat FSH at 1 and 3 days after MAA-treatment in comparison to stimulated control cultures (Fig 6.6). This pattern of response was observed irrespective of whether inhibin levels were measured after 24, 48 or 72h in culture (Figs 6.6, 6.7 and 6.8 respectively). Seminiferous tubules isolated from animals at 7 and 14 days after MAA-treatment and cultured in the presence of dibutyryl cyclicAMP or rat FSH secreted equivalent amounts of inhibin to that by respective controls after 24h in culture (Fig 6.6). However, inhibin levels in cultures of seminiferous tubules isolated from MAA-treated rats at 7 and 14 days post-treatment were significantly greater than respective controls after 48 and 72h in culture (Fig 6.7 and 6.8). The probable reason for this is that, while seminiferous tubules isolated from MAA-treated rats remained

Fig 6.5. Schematic representation of stage- and germ cell-specific effects of
MAA at successive intervals after treatment. Degenerating and/or absent germ
cells are represented by the areas enclosed within the shaded boxes (▨). Note
that the germ cells shown as absent or degenerating at 3 days after MAA
administration are taken directly from quantitative histological studies, whilst
those predicted as degenerating immediately after dosing (day 0) or at day 1
are deduced from these studies (Bartlett *et al*, 1988). Germ cell changes at 7,
14, 21 and 28 days post-treatment are calculated from the qualitative and
quantitative data presented by Bartlett *et al*, (1988) and Ratnasooriya &
Sharpe, (1989).

Time 0

1 Day

3 Days

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
(34.8)	(23.3)	(6)	(13)	(14.7)	(26.5)	(62.8)	(21.3)	(7.1)	(7.1)	(7.1)	(32.3)	(17.6)	(14.1)

Stages of the spermatogenic cycle.
(Duration of each stage (in hours) shown in brackets).

7 Days
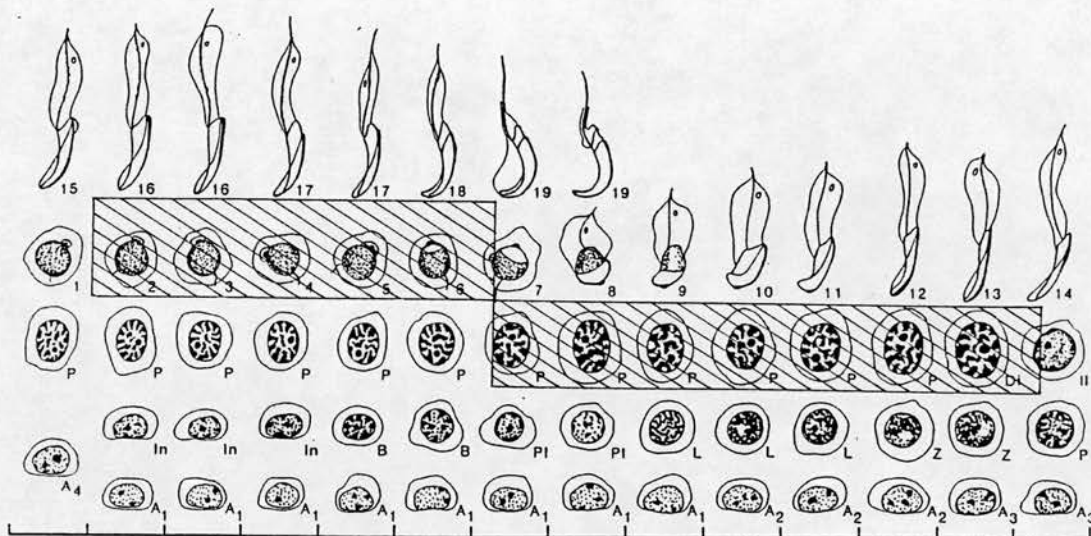
14 Days
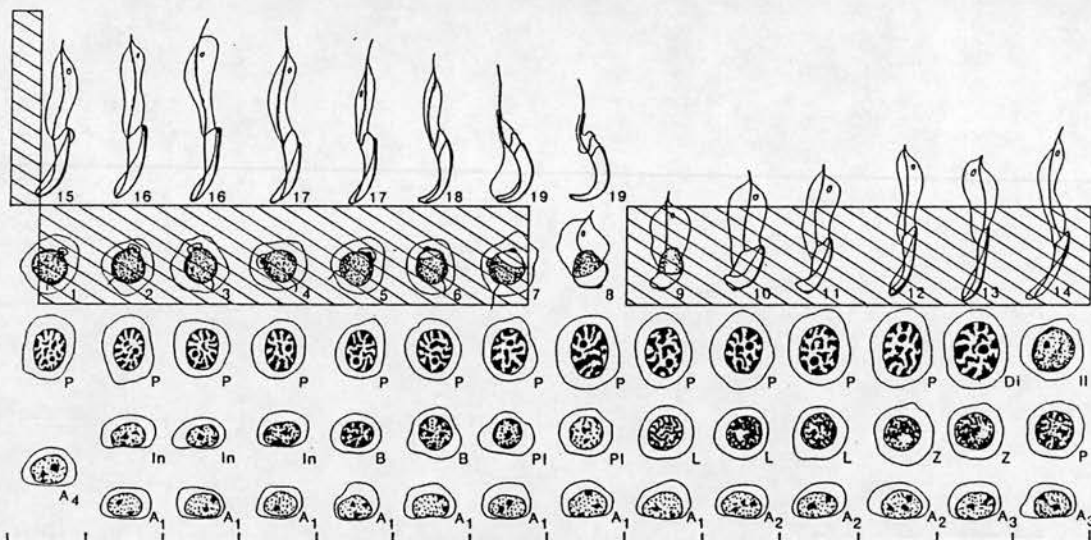
21Days
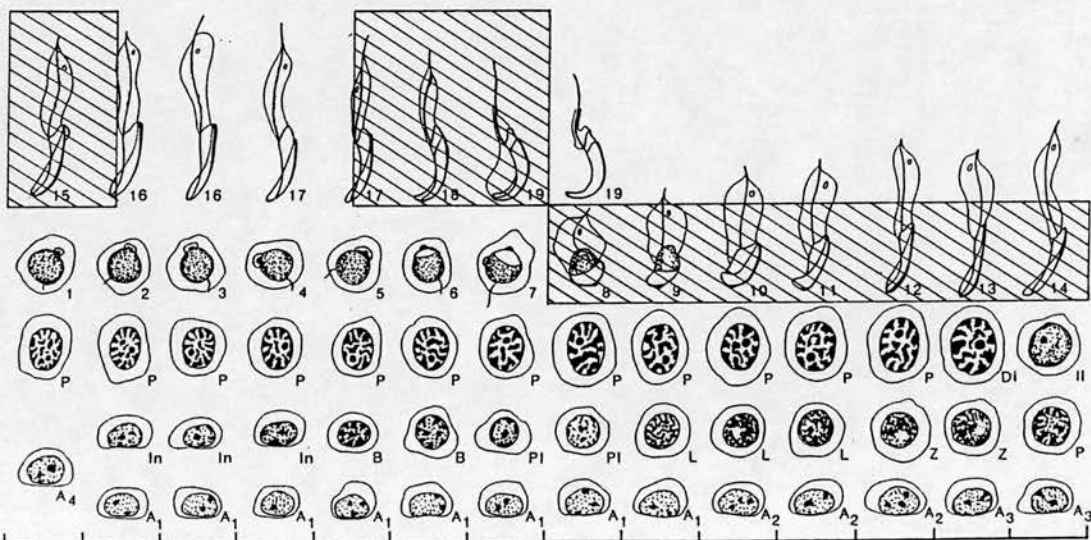
I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
(34.8)	(23.3)	(6)	(13)	(14.7)	(26.5)	(62.8)	(21.3)	(7.1)	(7.1)	(7.1)	(32.3)	(17.6)	(14.1)

Stages of the spermatogenic cycle.
(Duration of each stage (in hours) shown in brackets).

28 Days
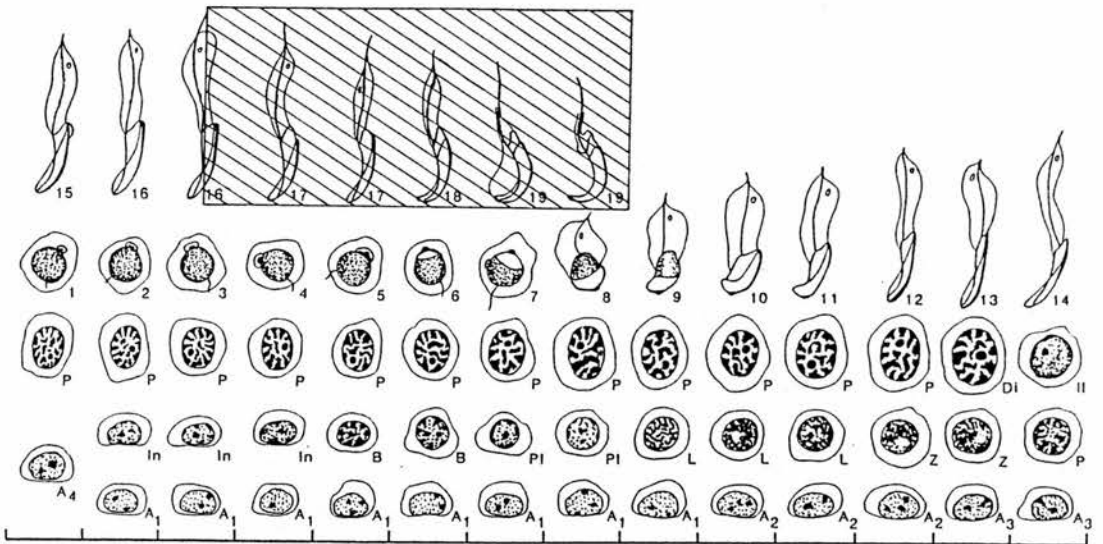
35 Days
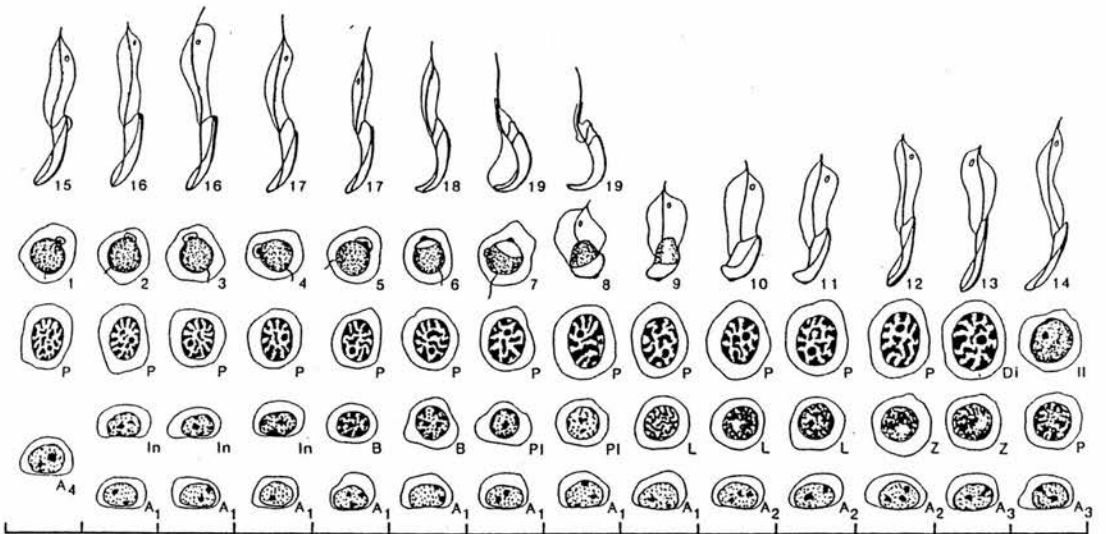
42 Days
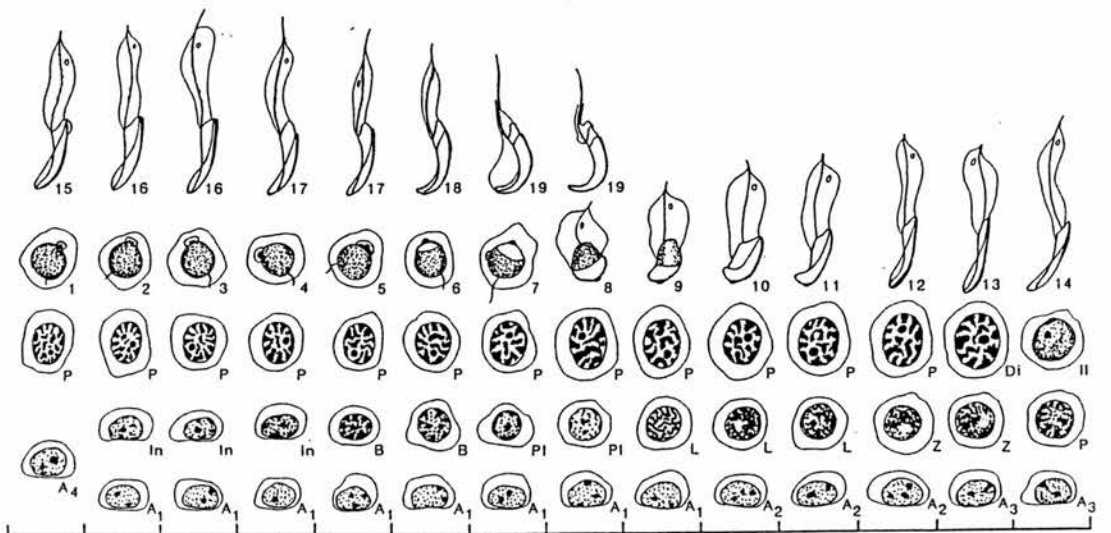
I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
(34.8)	(23.3)	(6)	(13)	(14.7)	(26.5)	(62.8)	(21.3)	(7.1)	(7.1)	(7.1)	(32.3)	(17.6)	(14.1)

Stages of the spermatogenic cycle.
(Duration of each stage (in hours) shown in brackets).

responsive to stimulation over the 72h period in culture, control cultures did not (Figs 6.6, 6.7 and 6.8). Therefore, control levels declined and, as a result, levels of inhibin secreted by seminiferous tubules cultured from MAA-treated rats were significantly higher than respective controls at 48 and 72h post-culture.
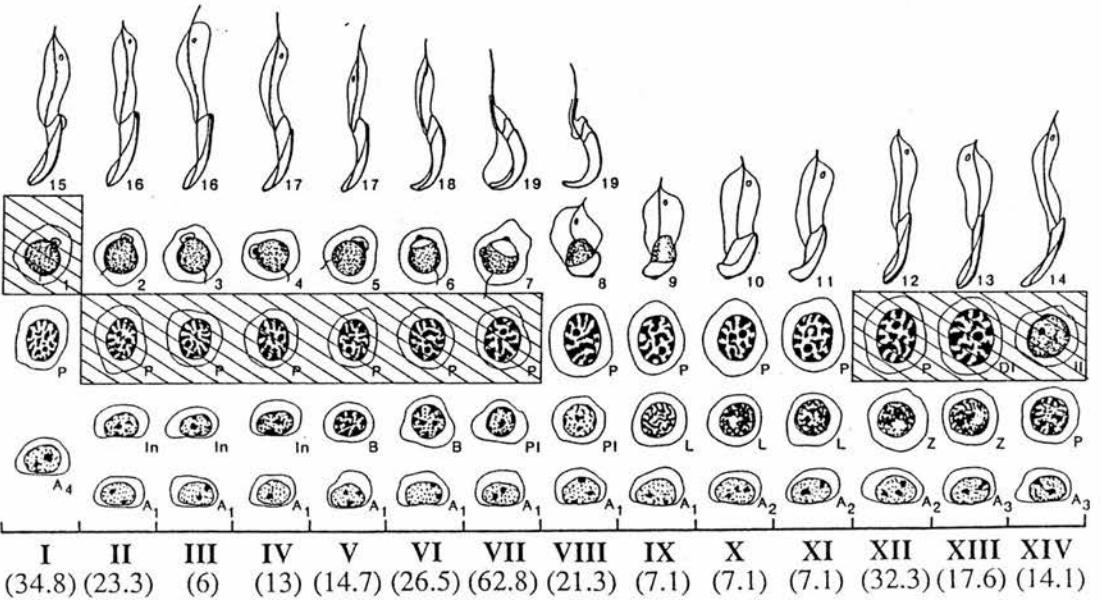
In parallel with the decrease in basal inhibin secretion from seminiferous tubules isolated at 21 and 28 days post-treatment, stimulated inhibin levels were also drastically reduced irrespective of whether measurements were made after 24, 48 or 72h in culture (Figs 6.6, 6.7 and 6.8 respectively). At the remaining time intervals after MAA-administration the stimulated levels of inhibin secreted by seminiferous tubules isolated from MAA- or saline- treated rats were not significantly different after 24h in culture (Fig 6.6). However, after 48 and 72h in culture, stimulated inhibin levels were significantly greater in cultures of seminiferous tubules derived from MAA-treated rats in comparison to saline treated controls (Figs 6.7 and 6.8). Again, this was probably due to a decrease in inhibin secretion by control cultures as a result of a decreased responsiveness with time in culture rather than to a direct effect of MAA post-isolation.

Further experiments were performed in a parallel study using an identical protocol but in which seminiferous tubules were isolated from different rats. The results obtained were remarkably similar to those obtained with the first set of cultures, results are shown in Figs 6.9, 6.10 and 6.11 respectively. Briefly, in these cultures, basal inhibin secretion at 1 and 3 days post-MAA treatment was increased significantly after culture for 24 (Fig 6.9), 48 (Fig 6.10) or 72h (Fig 6.11). In addition, at 21 and 28 days post-treatment, inhibin levels in medium conditioned by seminiferous tubules isolated from MAA-treated rats were reduced substantially over the 3 day culture period in comparison to seminiferous tubules isolated from vehicle-treated animals (Figs 6.9, 6.10 and 6.11 respectively). Therefore, the degree of inhibin stimulation produced by dibutyryl cyclicAMP and rat FSH and the changing pattern of inhibin secretion by seminiferous tubules isolated from MAA-treated rats were remarkably reproducible between animals and between experiments.

6.3.8. Effect of MAA adminstration *in vitro* on inhibin secretion by seminiferous tubules isolated from rats treated with vehicle alone.

Inhibin secretion by seminiferous tubules isolated from saline-treated rats at intervals after dosing and cultured in the absence or presence of 10^{-4} M MAA after 24, 48 or 72h in culture are shown in Figs 6.12, 6.13 and

Fig 6.6. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from one control (●) or MAA -treated rat (o) at various time intervals post-treatment. Data show inhibin secretion on day 1 of culture under basal conditions (a) or in the presence of db cAMP (b) or rat FSH (c). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown. Shaded areas at the top of the page represent schematically the type and extent of germ cell depletion induced by MAA *in vivo* at specific time intervals post-treatment, (PS = pachytene spermatocytes; RS = round spermatids; ES = elongate spermatids - see Bartlett *et al*, (1988) and Ratnasooriya and Sharpe, (1989).

Fig 6.7. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from one control (●) or MAA -treated rat (○) at various time intervals post-treatment. Data show inhibin secretion on day 2 of culture under basal conditions (**a**) or in the presence of db cAMP (**b**) or rat FSH (**c**). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown. Shaded areas at the top of the page represent schematically the type and extent of germ cell depletion induced by MAA *in vivo* at specific time intervals post-treatment, (PS = pachytene spermatocytes; RS = round spermatids; ES = elongate spermatids - see Bartlett *et al*, (1988) and Ratnasooriya and Sharpe, (1989).

Fig 6.8. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from one control (●) or MAA -treated rat (○) at various time intervals post-treatment. Data show inhibin secretion on day 3 of culture under basal conditions (**a**) or in the presence of db cAMP (**b**) or rat FSH (**c**). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown. Shaded areas at the top of the page represent schematically the type and extent of germ cell depletion induced by MAA *in vivo* at specific time intervals post-treatment, (PS = pachytene spermatocytes; RS = round spermatids; ES = elongate spermatids - see Bartlett *et al*, (1988) and Ratnasooriya and Sharpe, (1989).

Fig 6.9. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from different rats under identical conditions as those described in Fig 6.7. Data show inhibin secretion from one control (●) or MAA-treated rat (○) on day 1 of culture under basal conditions (**a**) or in the presence of db cAMP (**b**) or rat FSH (**c**). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown. Shaded areas at the top of the page represent schematically the type and extent of germ cell depletion induced by MAA *in vivo* at specific time intervals post-treatment, (PS = pachytene spermatocytes; RS = round spermatids; ES = elongate spermatids - see Bartlett *et al*, (1988) and Ratnasooriya and Sharpe, (1989).

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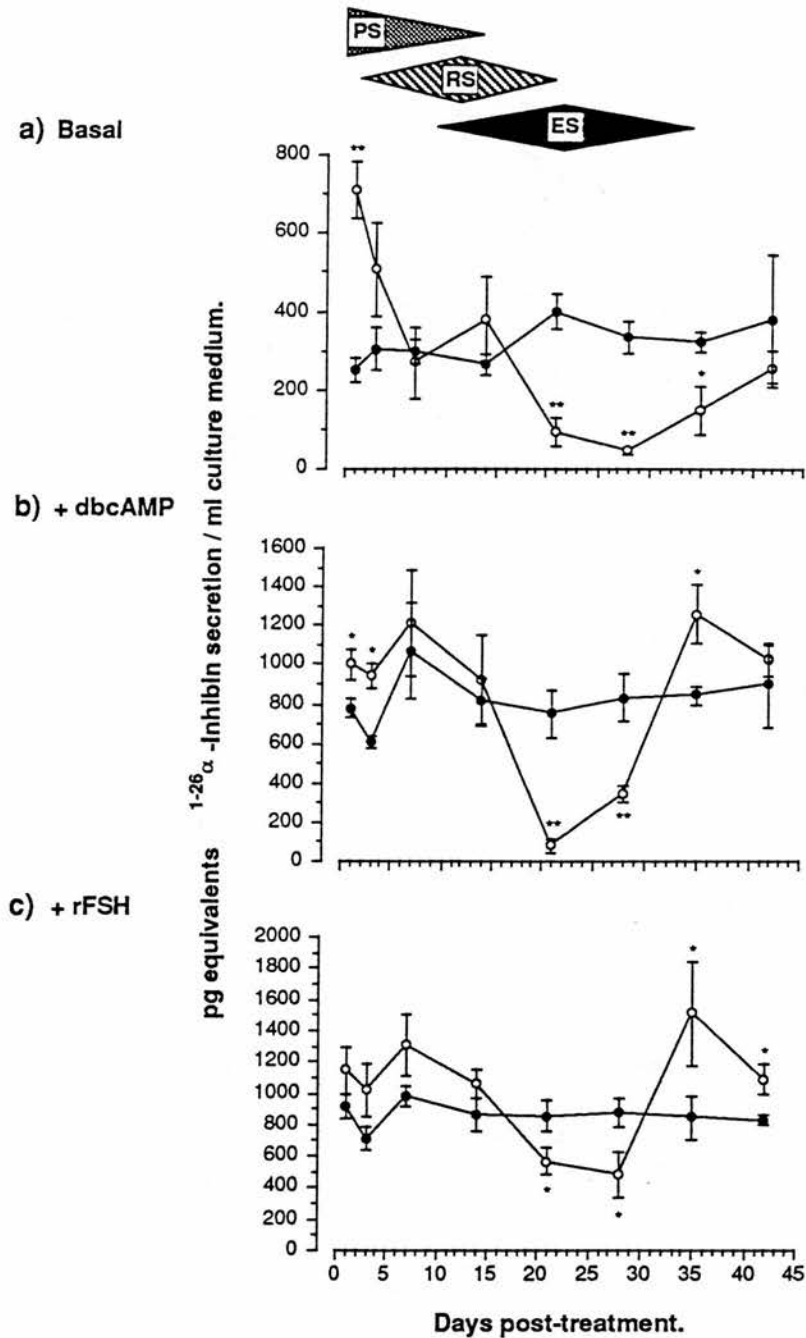
Fig 6.10.Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from different rats under identical conditions as those described in Fig 6.7. Data show inhibin secretion from one control (●) or MAA-treated rat (○) on day 2 of culture under basal conditions (**a**) or in the presence of db cAMP (**b**) or rat FSH (**c**). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown. Shaded areas at the top of the page represent schematically the type and extent of germ cell depletion induced by MAA *in vivo* at specific time intervals post-treatment, (PS = pachytene spermatocytes; RS = round spermatids; ES = elongate spermatids - see Bartlett *et al*, (1988) and Ratnasooriya and Sharpe, (1989).
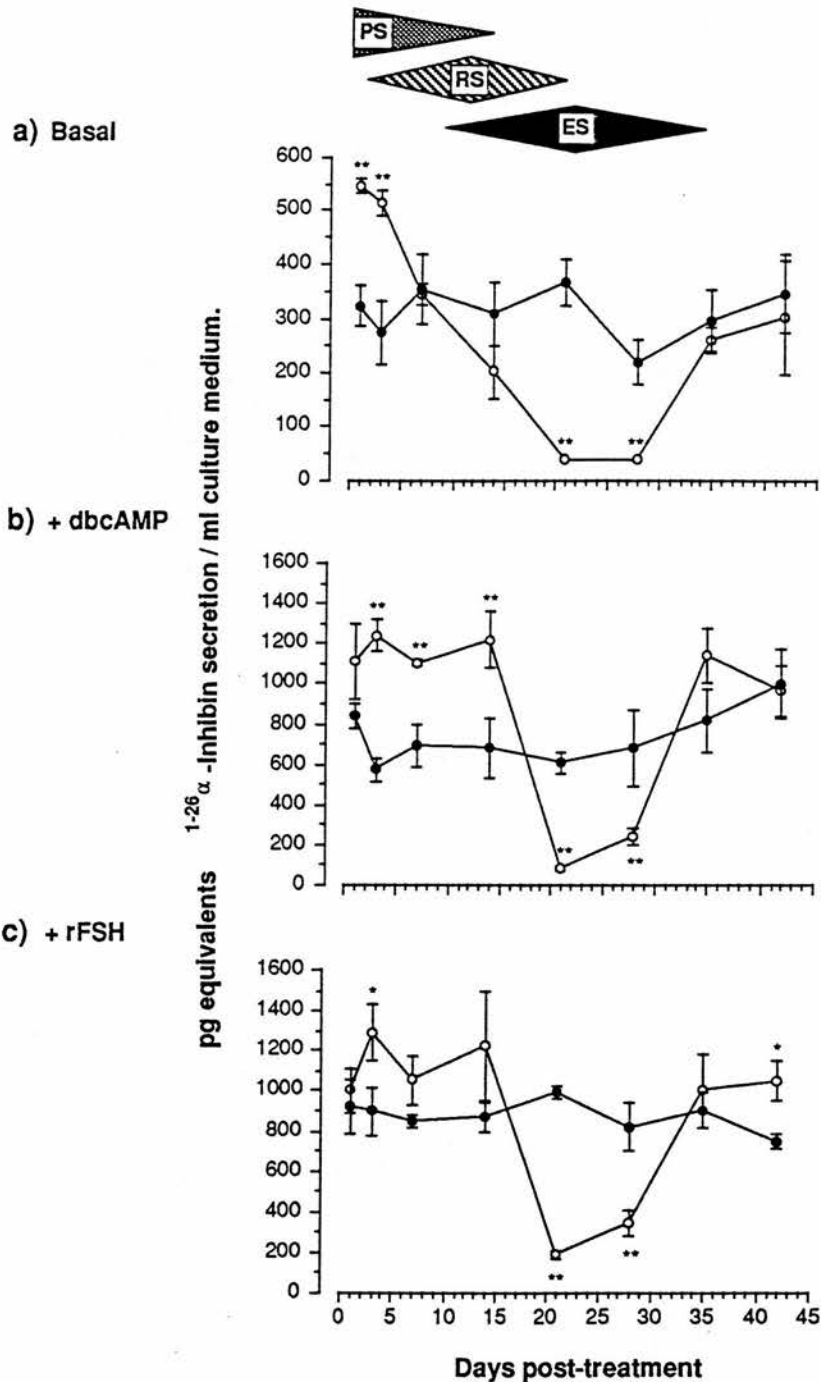
Fig 6.11. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from different rats under identical conditions as those described in Fig 6.7. Data show inhibin secretion from one control (●) or MAA-treated rat (○) on day 3 of culture under basal conditions (a) or in the presence of db cAMP (b) or rat FSH (c). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown. Shaded areas at the top of the page represent schematically the type and extent of germ cell depletion induced by MAA *in vivo* at specific time intervals post-treatment, (PS = pachytene spermatocytes; RS = round spermatids; ES = elongate spermatids - see Bartlett *et al*, (1988) and Ratnasooriya and Sharpe, (1989).
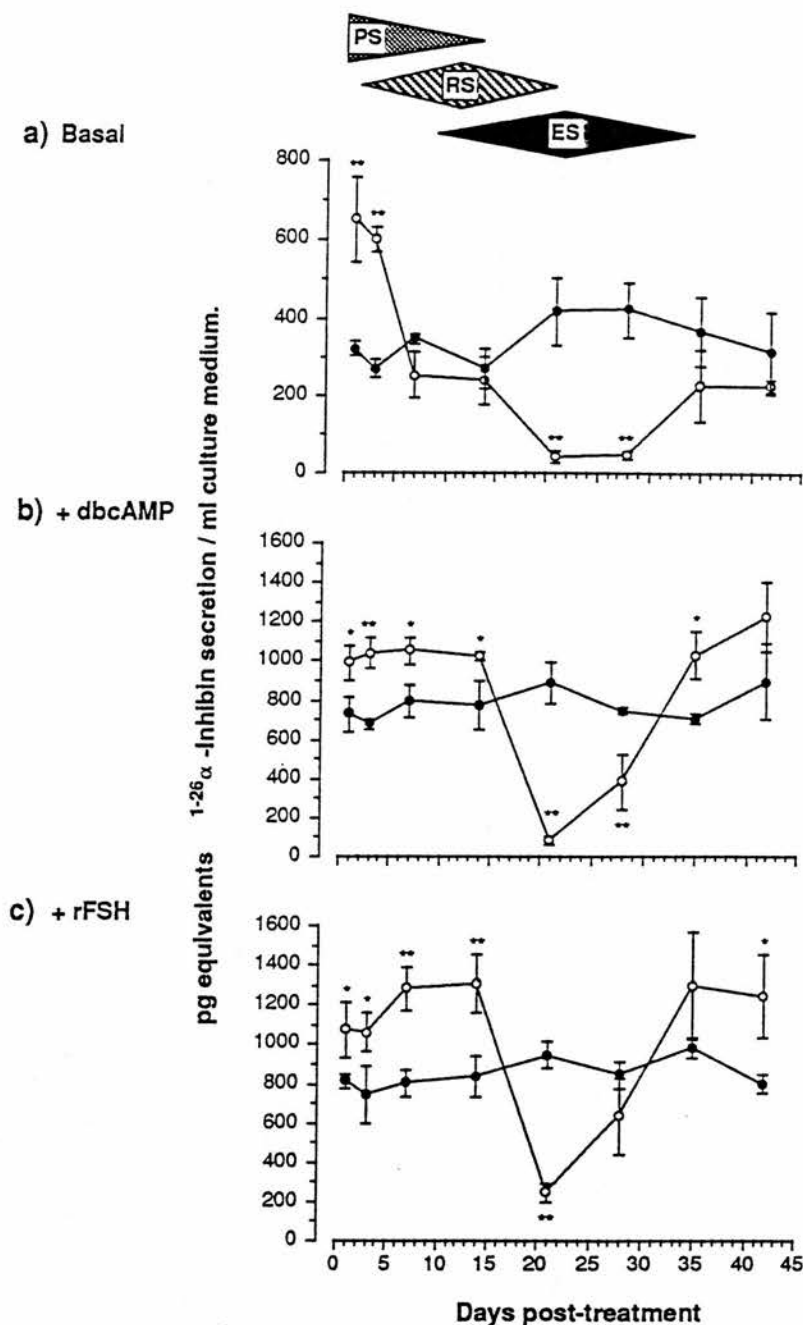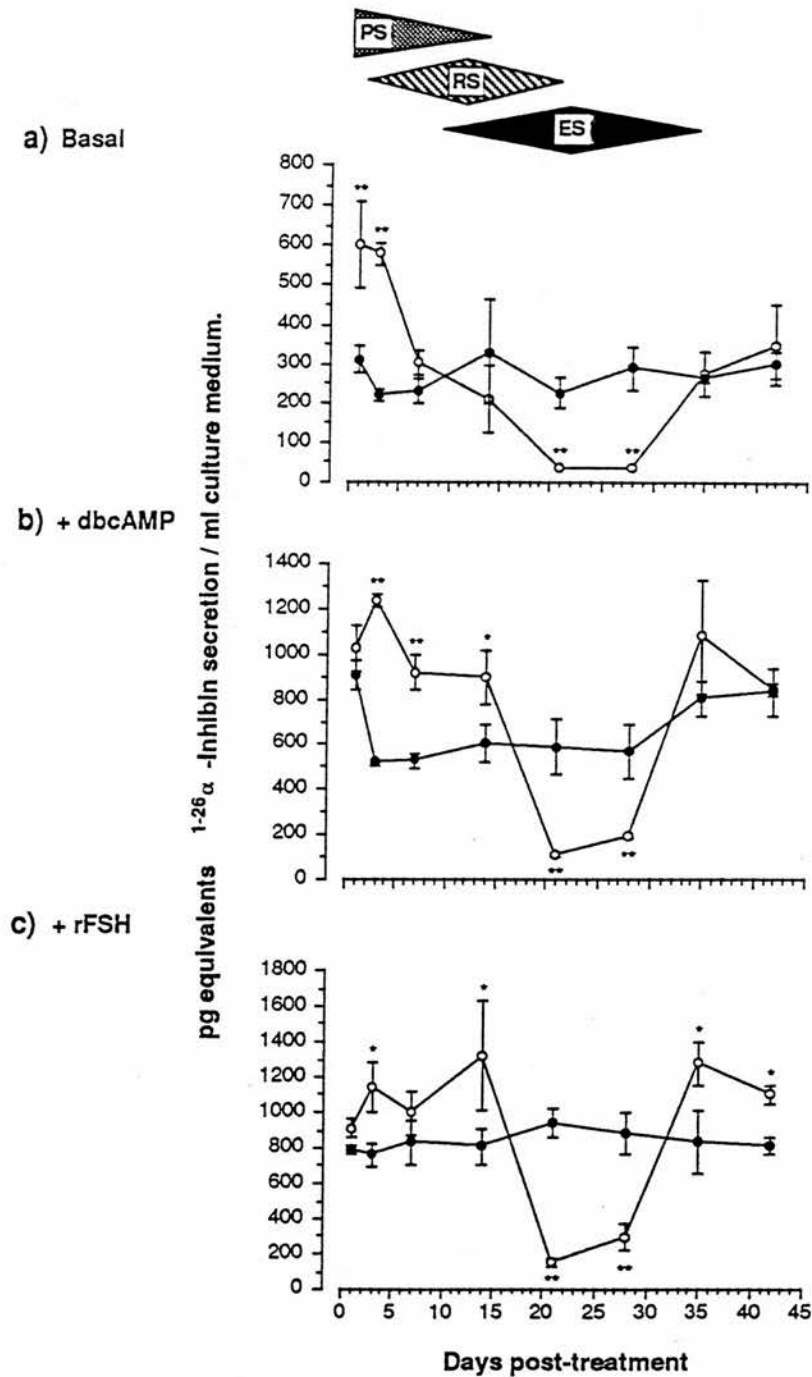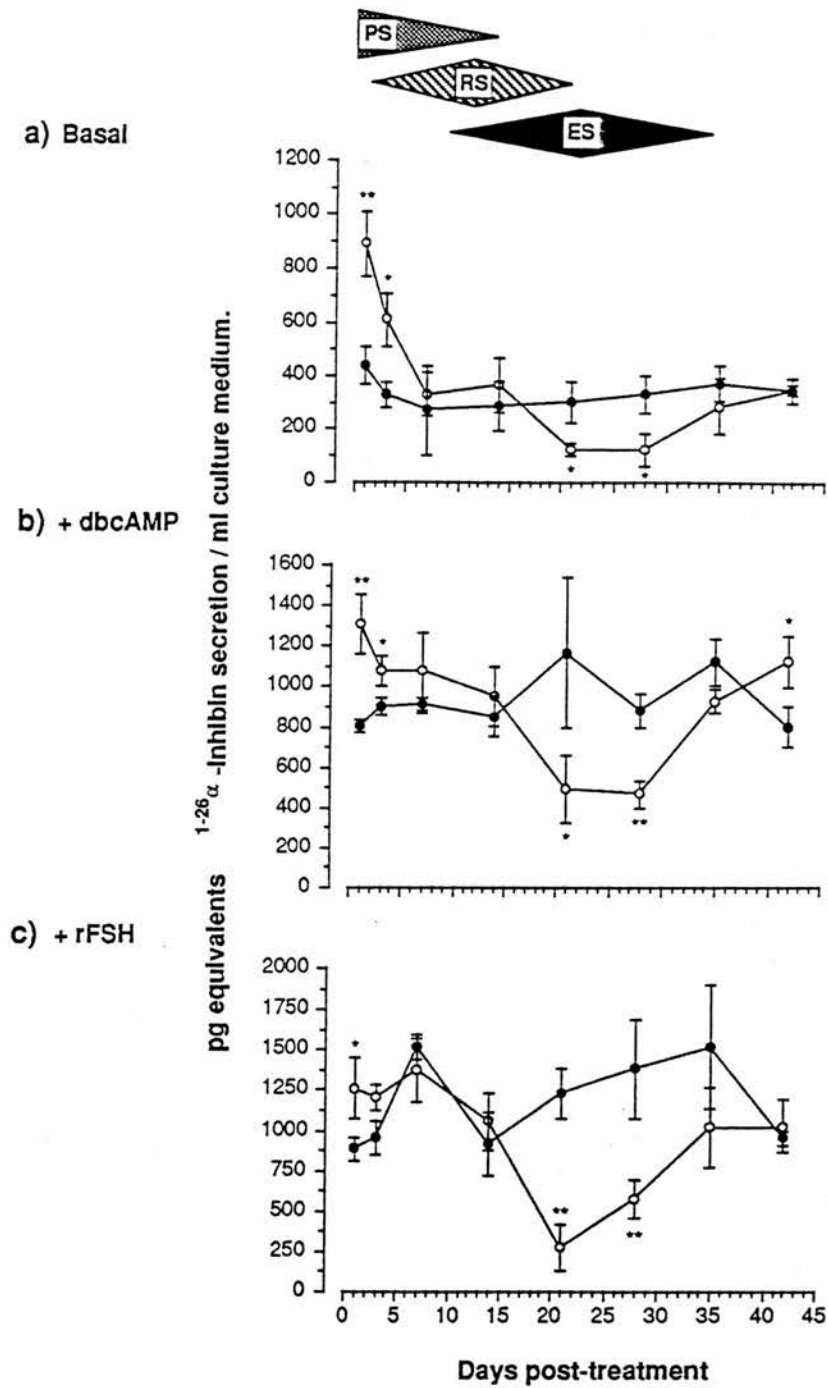
6.14 respectively. Results for a duplicate experiment using identical conditions but isolating seminiferous tubules from different animals are shown in Figs 6.15, 6.16 and 6.17.

The major effect of 10^{-4} M MAA *in vitro* was on basal inhibin secretion by seminiferous tubules isolated from saline-treated rats (Figs 6.12 a, 6.13 a and 6.14 a), whilst dibutyryl cyclicAMP- and rat FSH-stimulated inhibin secretion were relatively unchanged from control values (Figs 6.12 b, c, 6.13 b, c and 6.14 b, c). At all time intervals after MAA treatment higher basal inhibin levels were evident after culture for 24h in the presence of 10^{-4} M MAA, when compared to saline-treated controls (Fig 6.12 a). These differences were similar in magnitude after 48h in culture (Fig 6.13 a), but inhibin levels in the presence of MAA after 72h in culture had increased in comparison to inhibin secretion by comparable cultures after 48h as well as in comparison to inhibin secretion by control cultures at 72h (Fig 6.14 a). Although the responsiveness of tubules to both dibutyryl cyclicAMP and rat FSH was maintained in the presence or absence of MAA, there were no consistent or significant differences between inhibin levels in the presence or absence of 10^{-4} M MAA at 24 (Fig 6.12) or 48h (Fig 6.13) in culture. However inhibin levels in dibutyryl cyclicAMP-stimulated cultures after 72h in the presence of MAA were significantly raised at a number of time points when compared with equivalent controls (Fig 6.14).

The significant rise in basal inhibin secretion in the presence of 10^{-4} M MAA over 72h in culture was evident in a repeat experiment identical to that described above; results are shown in Figs 6.15, 6.16 and 6.17. Again after 24h, inhibin levels were raised significantly in the presence of MAA at a number of time points (Fig 6.15 a), whilst at 48 (Fig 6.16 a) and 72h (Fig 6.17 a) in culture, levels were significantly elevated at all time points. Although fluctuations in stimulated inhibin secretion in the presence or absence of MAA were detected and responsiveness to dibutyryl cyclicAMP and rat FSH were maintained in the presence of MAA under these stimulated conditions, the pattern of inhibin secretion in the presence or absence of MAA was comparable.

Furthermore, the concentration of MAA utilised in culture (10^{-4}M) was several orders of magnitude less than peak blood levels observed following a single oral dose of MAA at 650 mg/kg. These results therefore suggest that MAA has effects on inhibin secretion possibly through the perturbation of germ cells and/or Sertoli cells at concentrations significantly less than those evident *in vivo*.
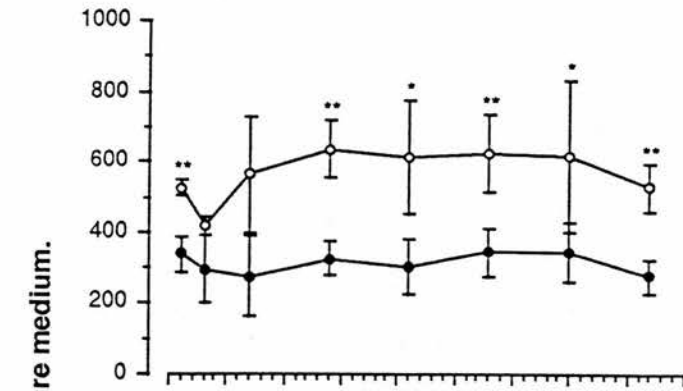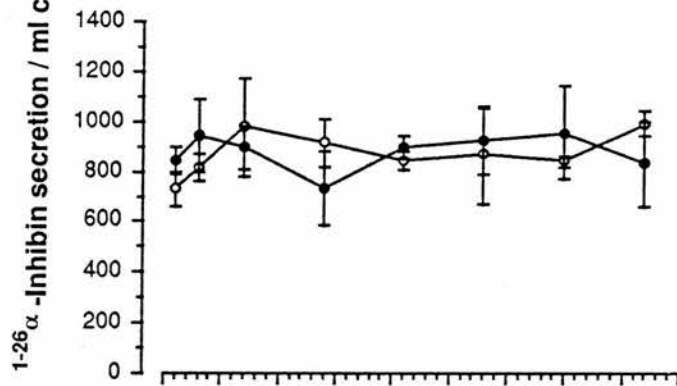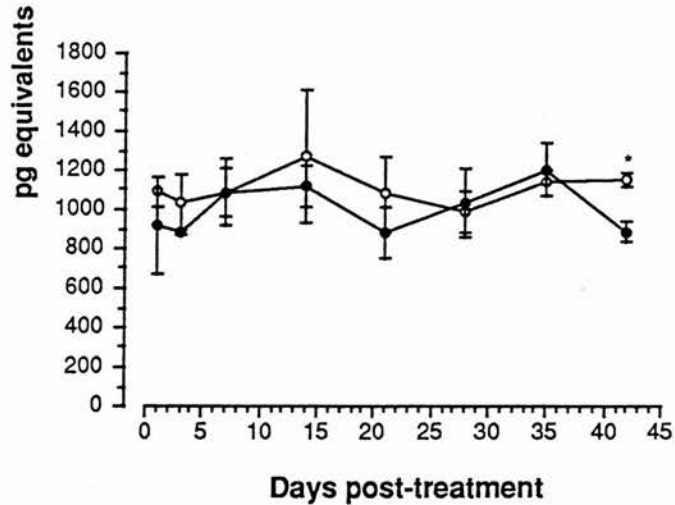
Fig 6.12. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from saline treated rats at various time intervals post-treatment and cultured in the absence (●) or presence (○) of 10^{-4} M MAA. Data show inhibin secretion on day 1 of culture under basal conditions (a) or in the presence of db cAMP (b) or rat FSH (c). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown.

a) Basal

b) + dbcAMP

c) + rFSH
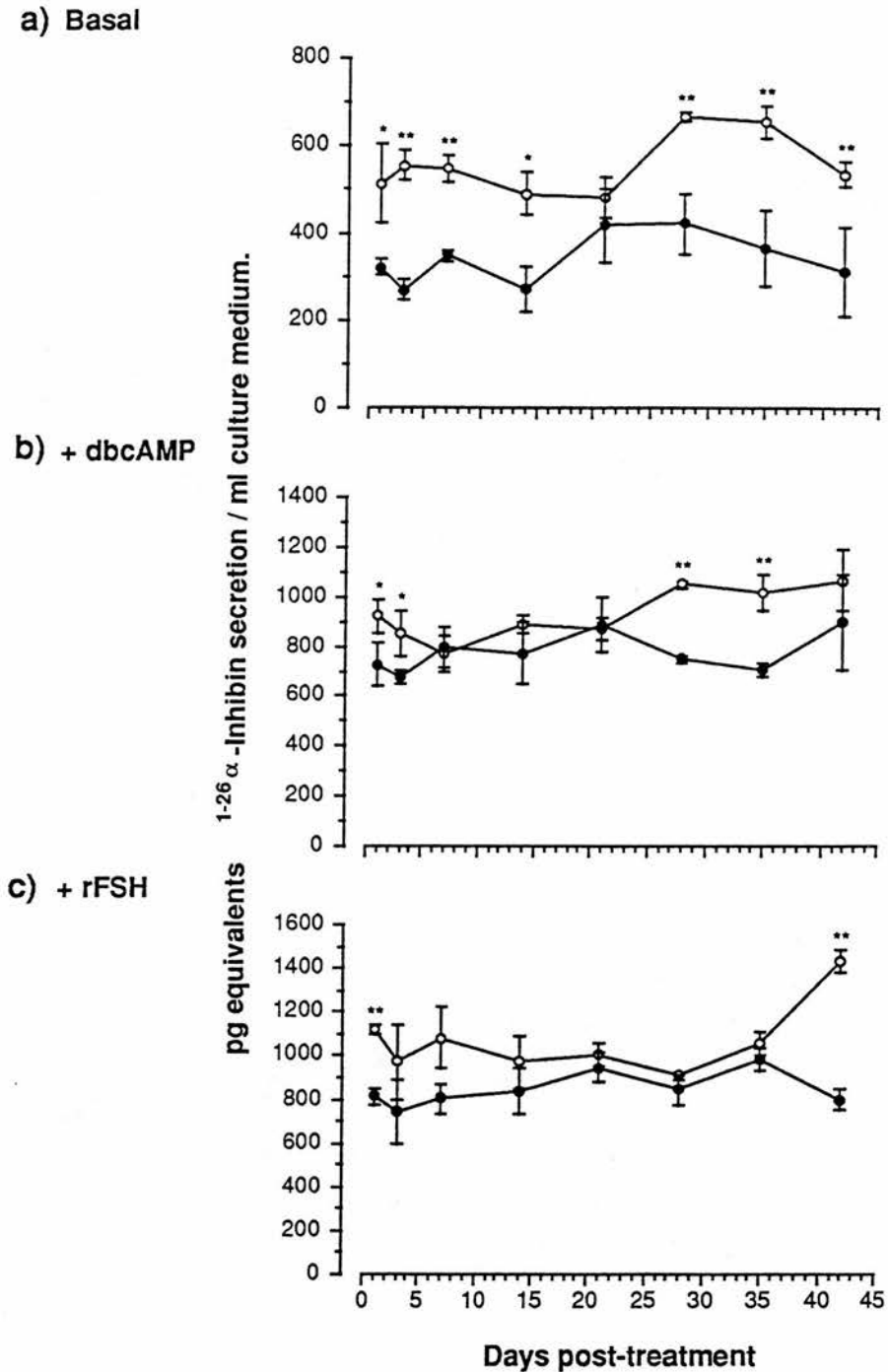
Fig 6.13. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from saline treated rats at various time intervals post-treatment and cultured in the absence (●) or presence (○) of 10^{-4} M MAA. Data show inhibin secretion on day 2 of culture under basal conditions (a) or in the presence of db cAMP (b) or rat FSH (c). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown.
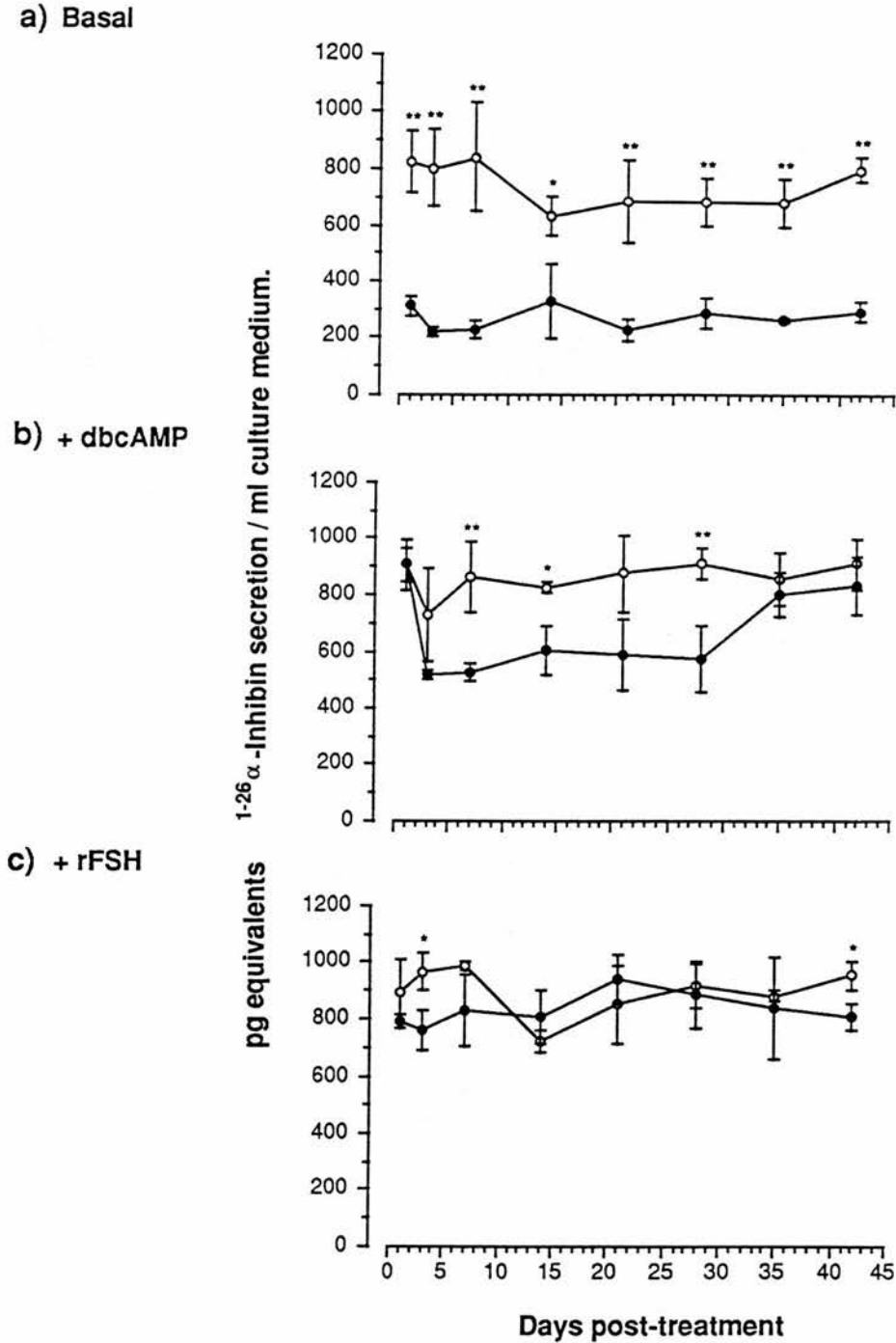
Fig 6.14. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from saline treated rats at various time intervals post-treatment and cultured in the absence (●) or presence (○) of 10^{-4} M MAA. Data show inhibin secretion on day 3 of culture under basal conditions (**a**) or in the presence of db cAMP (**b**) or rat FSH (**c**). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown.
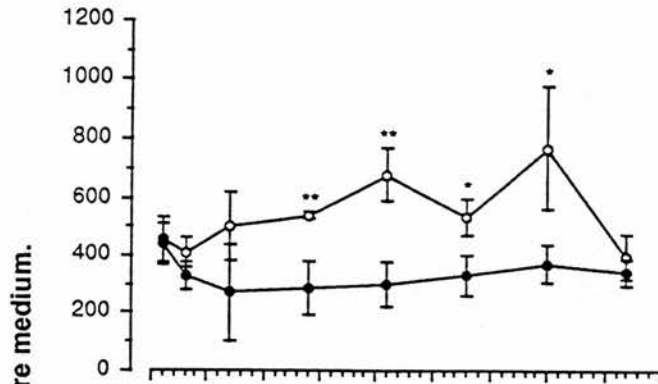
Fig 6.15. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from saline treated rats under identical conditions as those described in Fig 6.13. Data show inhibin secretion in the absence (●) or presence (○) of 10^{-4} M MAA on day 1 of culture under basal conditions (**a**) or in the presence of db cAMP (**b**) or rat FSH (**c**). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown.

Fig 6.16. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from saline treated rats under identical conditions as those described in Fig 6.13. Data show inhibin secretion in the absence (●) or presence (○) of 10^{-4} M MAA on day 2 of culture under basal conditions (a) or in the presence of db cAMP (b) or rat FSH (c). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown.
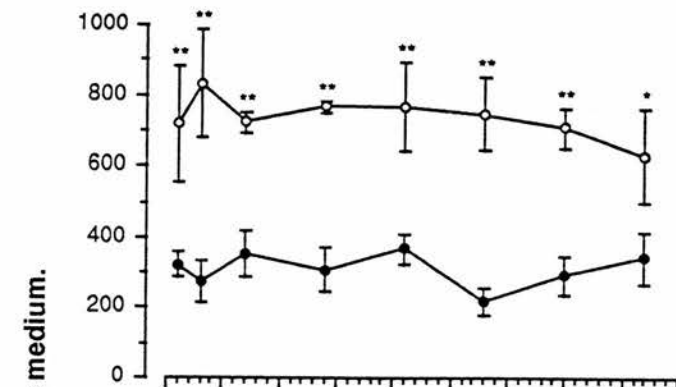
Fig 6.17. Secretion of immunoactive inhibin *in vitro* by seminiferous tubules isolated from saline treated rats under identical conditions as those described in Fig 6.13. Data show inhibin secretion in the absence (●) or presence (○) of 10^{-4} M MAA on day 3 of culture under basal conditions (**a**) or in the presence of db cAMP (**b**) or rat FSH (**c**). Values are means ± SD for 4 replicates (*P<0.01, **P<0.001, in comparison to respective controls). Results of a single experiment are shown.

6.4. DISCUSSION.

The primary objective of the present study was to investigate the possible role of selective germ cell types in the modulation of inhibin secretion by the Sertoli cell. Growing evidence exists to suggest that the secretory functions of the Sertoli cell are modulated by the presence or absence of specific germ cell types (Galdieri *et al*, 1981; Jegou *et al*, 1988; Le Magueresse, 1986; 1988; Le Magueresse & Jegou, 1986; 1988). To date, investigators have examined the role of germ cells in the secretion of other Sertoli cell derived factors using primary cell cultures and co-cultures which have numerous disadvantages, not the least of which is the destruction of the structural integrity of the seminiferous epithelium (discussed later). An alternative approach to the study of Sertoli cell function, particularly in the adult rat, is to isolate and culture seminiferous tubules. Furthermore, treatment of the animals using techniques designed to deplete specific germ cell types eg irradiation (Vihko *et al*, 1984; Pinon-Lataillade *et al*, 1988) or exposure to reproductive toxicants such as MAA (Sharpe *et al*, 1988) allows the potential regulatory effect of germ cells on Sertoli cell functions to be assessed using isolated seminiferous tubules while otherwise maintaining the structural integrity of the seminiferous epithelium.

MAA is an extremely useful research tool for inducing selective germ cell depletion, and thus to study the consequences in terms of changes in Sertoli cell function. These changes have been investigated *in vivo* (Bartlett *et al*, 1988). The toxicological, morphological and endocrinological effects of MAA and its parent compound ethylene glycol mono-methyl ether (EGME) have been described in detail in several studies (Foster *et al*, 1983; 1984; Chapin *et al*, 1984; Creasy *et al*, 1985; Bartlett *et al*, 1988; Ratnasooriya & Sharpe, 1989; Sharpe, 1989). Overall, the data show that MAA induces a highly selective effect on the testis, causing selective and stage-specific depletion of late zygotene spermatocytes in stage XII and pachytene spermatocytes in all stages except mid-stage VII (Bartlett *et al*, 1988). These effects are dose-dependent (Foster *et al*, 1983) with 650 mg/kg inducing the largest selective depletion of germ cells. As spermatogenesis proceeds near normally after treatment with only a small change in the duration of the spermatogenic cycle initially post-treatment (Chapin *et al*, 1984; Creasy *et al*, 1985), the major or complete (80-100%) depletion of pachytene and later spermatocytes 1 to 3 days post-treatment results in the absence of selective and predictable germ cell types through a process of maturation depletion at subsequent time intervals (Ratnasooriya & Sharpe, 1989). At later time intervals these are manifest as a depletion/loss of mainly round (days 7-14) or elongate (days 21-28) spermatids. This effect of selective

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germ cell depletion induced by MAA has been demonstrated to by very specific and highly reproducible (Bartlett *et al,* 1988; Ratnasooriya & Sharpe, 1989). While the effects of MAA on the hormonal milieu *in vivo* have been investigated (Bartlett *et al,* 1988) inhibin secretion *in vivo* was not measured. Furthermore the effects of MAA on basal and stimulated secretion *in vitro* were unknown.

In order to investigate modulation of Sertoli cell function by germ cells a number of experimental approaches have been adopted, most concentrating *in vitro.* The approach most widely used involves culturing Sertoli cells in the presence of selectively enriched populations of germ cells or medium conditioned by these selective germ cell types (Le Magueresse, 1988; Le Magueresse & Jegou, 1986). However there are a number of disadvantages to this culture technique for studying the influence of germ cells upon Sertoli cell function. Firstly, in order to generate the cultures, cell associations present *in vivo* must be destroyed and re-generated *in vitro*; the Sertoli cells are maintained alone in culture, germ cells are isolated, purified and added back to the Sertoli cell monolayer. Secondly, while it is possible to isolate and purify selective germ cell types (Le Magueresse *et al,* 1986; Pineau *et al,* 1990) their use is limited since only pachytene spermatocytes and round spermatids can be isolated in any great number and purity. Residual bodies have been used in an attempt to mimick in culture the effects of elongate spermatids on the Sertoli cell (Le Magueresse *et al,* 1986), but it is questionable whether this can be achieved given that elongate spermatids probably communicate normally with the Sertoli cell via elaborate functional complexes (Russell *et al,* 1983). In addition, Sertoli cell cultures are derived from the testes of immature rats. It is well established that the hormonal milieu within the immature male rat (especially with respect to inhibin and FSH levels) is markedly different from that in the adult animal (McLachlan, 1988; Maddocks & Sharpe, 1990). Therefore Sertoli cells isolated from immature animals are derived from a substantially different endocrine environment and, in the adult, interact with germ cell classes (e.g. round and elongate spermatids) which are more mature than those encountered *in vivo* prior to isolation.

Seminiferous tubule cultures have a number of advantages over primary cell cultures. Not only is the structural integrity of the seminiferous tubule maintained (at least initially) but the tissue can be derived from adult animals. Previous experimental work validating the culture system (see Chapter 2) has shown that tubules secrete inhibin in culture and remain responsive to rat FSH and dibutyryl cyclicAMP for a number of days. Therefore, using MAA-treatment, the interaction between selective germ cell types and the Sertoli cell in

the modulation of inhibin secretion was investigated *in vitro*. Tubules were isolated from animals at selected time intervals after MAA-induced depletion of germ cells from within the epithelium. These time intervals were selected based on previous data (Bartlett *et al*, 1988; Ratnasooriya & Sharpe, 1989) such that maturation depletion of successive classes of germ cell types occurred and the isolated tubules would therefore be depleted mainly of one germ cell class. Basal and stimulated inhibin secretion were measured in culture whilst hormone levels in serum and testicular IF were measured in other MAA-treated animals at these time intervals to correlate the hormonal profile evident *in vivo* with that observed *in vitro* with respect to inhibin secretion. However, it must be presumed that the *in vitro* secretion of inhibin by seminiferous tubules in culture reflects the summation of both apically and basally secreted inhibin by the Sertoli cell. The present study shows unequivocally that the ability of isolated seminiferous tubules to secrete immunoactive inhibin *in vitro* varies according to the germ cell complement and may reflect changes in hormone levels apparent *in vivo*.

In this study major changes were observed in the secretion of inhibin *in vivo*, and especially *in vitro*, by seminiferous tubules isolated from MAA-treated rats. As a number of factors could have contributed to these changes (e.g. alteration in FSH levels, in the bidirectional secretion of inhibin or in the germ cell complement), it is easier to, firstly, review briefly what is currently understood about the control of testicular inhibin secretion and then, secondly examine which of these possibilities might apply to the present findings.

Firstly, numerous studies have postulated a relationship between FSH and inhibin, especially peripheral blood levels but also a relationship between serum FSH and IF levels of inhibin. Historically, inhibin has been postulated to negatively regulate FSH secretion (de Jong, 1988) although more recent studies have demonstrated more of a positive relationship between FSH and inhibin levels in the blood of adult animals and men (McLachlan, 1988). Indeed in this study using isolated seminiferous tubules or in other studies using primary cell cultures (see Chapter 4 and Biscak *et al*, 1987) the administration of FSH or dibutyryl cyclicAMP increased inhibin secretion in a dose-dependent manner. Previous studies have demonstrated that the levels of peripheral FSH and inhibin are significantly elevated in the immature male rat but decline together during puberty and into adulthood (Sharpe & Maddocks, 1990). The current interpretation is that, in the rat at least, inhibin is more important in negatively regulating FSH levels in the truly prepubertal animal (ie 10-20 days

old) than at puberty and later. Recently, Maddocks & Sharpe, (1989; 1990) have demonstrated that peripheral blood inhibin is derived predominanly from inhibin secreted into seminiferous tubule fluid and which is then resorbed via the mediastinal venous plexus which overlies the rete testis. Other studies (Sharpe & Maddocks, 1989) have demonstrated that IF inhibin levels, in the adult rat, frequently bear little relationship to peripheral inhibin levels.

Secondly, inhibin is secreted in a bidirectional manner by Sertoli cells (Sharpe *et al*, 1988; Maddocks & Sharpe, 1990), with probably more being secreted into seminiferous tubule fluid than IF in the normal adult testis (Maddocks & Sharpe, 1989; 1990). Changes in the bidirectional secretion of inhibin occur with age and after hormone treatment (hCG) or other treatments which alter serum FSH levels. Indeed in nearly every instance where FSH levels increase, IF levels of inhibin also rise - the exception being in severely atrophied testes, such as the cryptorchid testis (Sharpe *et al*, 1988). During puberty there is a very close positive correlation between IF and peripheral plasma levels of inhibin (perhaps because a substantial proportion of peripheral inhibin comes from that in IF via testicular venous blood). In the adult, the relationship is not so clear cut although clearly when IF levels of inhibin are increased after hCG treatment, so are testicular venous and peripheral plasma levels (Maddocks & Sharpe, 1990). However, perhaps such a clear-cut relationship should be expected in the adult rat where IF inhibin is a relatively minor determinant (approximately 5-20%) of peripheral inhibin levels at least via testicular venous blood (Maddocks & Sharpe, 1990). In addition a small proportion will also be returned to the peripheral blood via lymphatic drainage.

Thirdly, a number of previous studies *in vivo* and *in vitro* have demonstrated that selective germ cell types modulate a number of Sertoli cell functions such as the secretion of androgen binding protein (Galdieri *et al*, 1981; Gunsalus *et al*, 1981; Le Magueresse *et al*, 1986; Le Magueresse & Jegou, 1986), plasminogen activator and transferin (Griswold *et al*, 1988), and a number of Sertoli cell-derived proteins with as yet unidentified functions. Further evidence is derived from; 1) the study of the morphological and biochemical changes in Sertoli cells in relation to the stages of the cycle of the seminiferous epithelium (Parvinen, 1982); 2) the addition of germ cells or germ cell conditioned media to immature Sertoli cells in culture (Galdieri *et al*, 1984; Le Gac *et al*, 1983; Le Magueresse *et al*, 1986; 1988; Le Magueresse & Jegou, 1986; 1988; Ireland & Welsh, 1987); 3) the destruction of germ cells by a variety of treatments *in vivo* and the study of its consequences on Sertoli cell function (Jegou *et al*, 1983; 1984; Vihko *et al*, 1984; Pinon-Lataillade *et al*,

1988). With respect to germ cell modulation of Sertoli cell inhibin secretion, previous studies performed using Sertoli cell cultures and co-cultures have demonstrated that basal or FSH-stimulated inhibin secretion is enhanced by up to 2- to 3- fold in the presence of germ cells (see Chapter 4). Furthermore, recent experimental evidence suggest that in primary culture selective germ cell classes (primarily early spermatids) when isolated, purified and added back to Sertoli cell monocultures enhance levels of the mRNA for the α-subunit of inhibin as well as the secretion of immunoactive and bioactive inhibin (Pineau *et al,* 1990).

In the present study, opposite changes in inhibin secretion occurred *in vivo* at 1 and 3 days post-treatment compared with that at 21 to 35 days after MAA administration. During both of these periods FSH levels were increased and at both intervals there was major depletion of germ cell classes; primarily pachytene spermatocytes during the former period and elongate spermatids during the latter. Since IF inhibin levels were increased at 1 and 3 days post-treatment but were unchanged at later time intervals post-treatment, and since no real difference between testis weights at either of the time periods was evident, (therefore suggesting that this situation is dissimilar to that observed in the case of the cryptorchid testis), then the repeatable pattern of change in IF inhibin *in vivo* could be explained by one, or a combination of the following explanations.

Firstly, at 1 and 3 days after MAA-treatment serum FSH levels were raised significantly in the present study, confirming the findings of Bartlett *et al,* (1988). Previous studies have shown that in several situations levels of inhibin in IF increase proportionately in the presence of increased levels of serum FSH (Sharpe *et al,* 1988; Sharpe & Maddocks, 1989; Maddocks & Sharpe, 1989). However, this would not explain the absence of raised inhibin levels in testicular IF when serum FSH levels were significantly elevated between 21 and 35 days post-treatment. Therefore, rises in serum FSH are not solely responsible for increases in IF inhibin levels but may be a contributory factor particularly at the earlier time-points.

A further explanation for the lack of rise in IF inhibin levels between 21 and 35 days post-treatment may lie in a change in the route of secretion of inhibin from the Sertoli cell and, ultimately, from the testes into the peripheral circulation. If the increases in serum FSH levels observed at either 1 to 3 or 21 to 35 days post-treatment are due to decreased negative feedback by inhibin, through a reduction in peripheral blood levels of inhibin, then this would indicate (indirectly) that inhibin secretion into the circulation in the adult

rat is via seminiferous tubule fluid through the mediastinal venous plexus (see above). As the levels of inhibin in IF were unchanged between 21 and 35 days post-treatment, (in comparison to those levels of inhibin in IF observed between 1 and 3 days post-treatment), this would mean that there was a proportionated change in the route of secretion of inhibin. As yet no direct evidence to support this hypothesis has been obtained. The measurement of peripheral inhibin levels would resolve this situation; unfortunatly due to problems in the inhibin RIA in measuring inhibin levels in serum, peripheral blood levels of inhibin could not be measured in this study.

Bartlett and co-workers (1988) in a similar *in vivo* study to that used presently, also demonstrated a significant increase in serum FSH levels at 21 days post-treatment. Furthermore in this study significant increases in serum LH levels were evident at 21 and 28 days post-treatment. Thus the increase in serum FSH may be the result of a decrease in steroid negative feedback and the increase in serum levels of LH at 21 and 28 days may be a reflection of 'compensated Leydig cell dysfunction' ie higher than normal LH levels are required to maintain normal blood levels of testosterone. Bartlett *et al* (1988) also showed an increase (although not significant) in serum LH levels at 21 days post-treatment. Serum and intra-testicular testosterone levels, however, did not vary in MAA-treated rats at 21 and 28 days post-treatment. This is not surprising since testosterone insufficiency may have already occurred and a compensatory increase in LH taken place between 14 and 21 days. There is indirect evidence for marginal testosterone insufficiency at 21 days after MAA-treatment, judging from the increased degeneration of pachytene spermatocytes and round spermatids at stage VII compared with controls (Ratnasooriya & Sharpe, 1989). Such changes are classical signs of androgen insufficiency (Sharpe *et al*, 1990), although based on studies of the dose-response relationship between testosterone levels and germ cell degeneration at stage VII, it would have to be concluded that the testosterone insufficiency in MAA-treated rats was only marginal. However, this would fit with the observed lack of change in testosterone levels themselves, which are always very variable - hence only large changes can ever be detected. Another more likely explanation for the dichotomy in results for inhibin and FSH levels observed at the earlier and later time intervals after MAA-treatment could be the result of changes in the germ cell complement. If this is the case then the results suggest that pachytene spermatocytes and elongate spermatids (but not round spermatids) are involved in the control of inhibin secretion. There is very good evidence from other studies (Pinon-Lataillade *et al*, 1988) to suggest that elongate spermatids may

control inhibin secretion and in all situations where there is specific depletion of elongate spermatids, serum FSH levels increase as in the present study. Therefore this would seem to be the most likely explanation for the decreased secretion of inhibin at 21 days post-treatment, especially as inhibin secretion returned to normal from 28 to 42 days post-treatment in parallel with the restoration of elongate spermatid numbers.

Although the results observed *in vivo* or ex-*vivo* over the first 3 days post-treatment could indicate a negative controlling effect of pachytene spermatocytes on Sertoli cell inhibin secretion, there are other, perhaps more logical explanations: Firstly, FSH levels and IF inhibin levels were both increased at 1 and 3 days post-treatment *in vivo*, which therefore could be due to a direct effect of FSH on the Sertoli cell. This hypothesis is supported by the finding *in vitro* that basal inhibin secretion in culture was raised to approximately the same level as that induced by FSH and/or dibutyryl cyclicAMP. Secondly, the rise in inhibin levels *in vivo* at 1 and 3 days post-treatment may be a direct effect of MAA on Sertoli cell inhibin secretion. Tissue distribution studies performed *in vivo* have demonstrated that, following a single oral dose of MAA at 650 mg/kg, a peak testicular concentration of 5×10^{-3}M MAA is attained within 24h of treatment (Foster - personal communication). The increase in basal inhibin secretion in culture observed at 24, 48 or 72h in the presence of 15- fold less MAA may be a reflection of the changes observed *in vivo* between 1 and 3 days post-treatment and those evident in culture after the isolation of seminiferous tubules from rats pre-treated 1-3 days previously with MAA (Figs 6.6 to 6.11). This may mean that MAA has effects *in vitro* similar to those observed *in vivo* (in terms of inhibin secretion) but at lower doses, and that MAA directly (via an effect on the Sertoli cell) or indirectly (via the degeneration of pachytene spermatocytes) increases total inhibin secretion by Sertoli cells progressively over a 3 day culture period. However it remains to be determined if MAA causes these effects on inhibin secretion through an increase in cAMP production or via another pathway, possibly involving changes in Sertoli cell lactate and/or pyruvate secretion. Previous studies have demonstrated that MAA has effects on the secretion of these factors by primary co-cultures of Sertoli cell and germ cells (Williams & Foster, 1988). MAA selectively increases Sertoli cell lactate production, probably through the inhibition of LDH-C4, and causes germ cell exfoliation (initially pachytene spermatocytes) in co-culture. Inhibin levels in primary cultures and co-cultures in the presence of MAA have yet to be measured. It is also possible that some changes occur within seminiferous tubules *in vitro* and

it is this pachytene spermatocyte loss which results in increased secretion of inhibin in culture. There is no evidence to refute this possibility and all of the *in vitro* and *in vivo* data is consistent with this possibility. Detailed studies of the morphology of cultured seminiferous tubules in the presence or absence of MAA will be necessary to answer this question or the use of some other testicular toxicant to selectively reduce/remove pachytene spermatocytes.

The present *in vitro* data shows unequivocally that elongate spermatids, particularly at the later stages of development, modulate the secretion of inhibin. The control of Sertoli cell function by specific complements of germ cells is now well documented and elongate spermatids have been implicated in several studies as controlling a number of Sertoli cell functions (Pinon-Latillade *et al*, 1988; Jegou *et al*, 1988; Sharpe, 1989). Previous studies have suggested that Sertoli cell inhibin production is controlled by specific complements of germ cells (Pineau *et al*, 1990).

In this study at 21 and 28 days after MAA administration, maturation depletion of elongate spermatids had occurred *in vivo* such that seminiferous tubules isolated from animals at these time points were denuded of most elongate spermatids at 21 days and of step 16 to 19 elongate spermatids at 28 days post-treatment (see Fig 6.5.). In culture, significantly less inhibin was secreted by seminiferous tubules isolated at these time points post-treatment in comparison to cultures derived from saline-treated controls, both under basal and stimulated conditions, raising the possibility of paracrine control of Sertoli cell inhibin secretion by elongate spermatids.

In conclusion, the present study has used MAA as a biochemical tool to selectively deplete the seminiferous epithelium of germ cells to study testicular paracrine interactions possibly involved in the modulation of inhibin secretion *in vivo* and *in vitro* by adult Sertoli cells. Measurement of basal and stimulated inhibin secretion by isolated seminiferous tubules following MAA administration provides unequivocal evidence that elongate spermatids, (and possibly pachytene spermatocytes), modulate adult Sertoli cell inhibin secretion. Further use of this compound may help to elucidate the complex interplay between Sertoli cells, Leydig cells and germ cells in the control of spermatogenesis.

Chapter 6 - Notes.

CHAPTER 7.

GENERAL DISCUSSION.

7.1. GENERAL DISCUSSION:

The principle aim of the studies in this thesis was to evaluate the potential use of known and/or suspected testicular toxicants with different sites of action as biochemical tools with which to dissect the normal Sertoli cell - germ cell interactions that form part of the foundations of spematogenesis. These testicular toxicants have, to a greater or lesser extent, been evaluated by previous investigations *in vivo* and/or *in vitro* from a toxicological perspective. Studies presented here are among the first to evaluate these compounds as "biochemical tools" to investigate spermatogenesis. The compounds utilised were *meta* dinitrobenzene (mDNB) and nitrobenzene (NB), which are known or suspected Sertoli cell toxicants respectively, methoxyacetic acid (MAA), a toxicant which induces the degeneration of pachytene spermatocytes and the isomers of mononitrotoluene (mNT) which have been previously reported to cause testicular disruption through an unknown mechanism and site of action. As a "spin off", these studies may help to identify the site and mechanism of action of testicular toxicants and obviously aid in the evaluation of hazard assessments of other suspected toxicants and in the design of possible non-toxic alternatives.

The primary aim of the project was encapsulated within the serendipitous finding that elongate spermatids modulate inhibin secretion by Sertoli cells. These results were demonstrated using cultures of seminiferous tubules. The technique of dissecting and culturing lengths of seminiferous tubules following treatment *in vivo* or *in vitro* with MAA represents a potentially exciting and relatively simple method for studying Sertoli cell - germ cell interactions *in vitro*, (or ex-*vivo*) in a relatively intact epithelium i.e. missing one class of germ cells. The technique has widespread applications which are discussed later.

Secondary aims of the present study were: Firstly to further investigate and assess the use of *in vitro* systems (Sertoli cell monocultures, Sertoli-germ cell co-cultures or isolated seminiferous tubules) as models for studying the mechanism and site of action of testicular toxicants which perturb the seminiferous epithelium. Secondly, to evaluate for the first time the potential of inhibin as a marker of toxicant action on the testis.

The nitroaromatic compounds comprise an economically important class of chemical used in the manufacture of several thousand consumer products. Estimates of as much as 10% of sales by the chemical industry are based on processes which, at some step, involve nitroaromatic compounds. Previous investigators have demonstrated that mDNB is a testicular toxicant. Both *in vivo* and *in vitro* studies have shown that it induces toxicity

via a direct effect on the Sertoli cell. The studies presented here have demonstrated in detail the temporal changes in testicular histology following mDNB administration and the degree of reversibility post-treatment, results which are in agreement with data published during the course of this work (Hess *et al*, 1988; Rehnberg *et al*, 1988). In addition, the effect of perturbation of the Sertoli cell by mDNB and its relationship to the testicular-pituitary hormonal axis were examined. mDNB induced significant changes in the levels of FSH and inhibin at early time intervals post-treatment. While rises in the former are known to be an insensitive indicator of massive germ cell degeneration, the measurement of the latter (inhibin), adds to the growing evidence that the levels of FSH and inhibin are positively correlated *in vivo*. Inhibin represents a Sertoli cell secretion present in peripheral plasma and therefore could be a possible biochemical marker of testicular function. The identification of significant increases in inhibin secretion in Sertoli cell mono- and co-culture, in the presence of mDNB at equivalent concentrations to those found in testicular interstitial fliud following treatment, suggest that events observed *in vivo* are reproducible *in vitro*. Furthermore these increases in inhibin secretion observed *in vitro* occurred at doses which have been demonstrated in these and others studies (Williams & Foster, 1988) as toxic using additional indices of Sertoli cell function including germ cell exfoliation, morphology in culture and lactate and pyruvate secretion.

mDNB may therefore represent a viable chemical alternative to the physical techniques, (cryptorchydism, X-irradiation and heat treatment) already employed to disrupt testicular function *in vivo* in order to study normal versus disrupted spermatogenesis, but mDNB has the additional advantage that it specifically perturbs Sertoli cell function. The primary disadvantage to the use of mDNB in this manner is the variability in its effects between animals. From a retrospective viewpoint, doses in excess of 25 mg/kg (possibly 48 mg/kg - Blackburn *et al*, 1988) should have been employed which would possibly reduce the problems with variability of effect. However, although the degree of variability could have been reduced it would never have been completely abolished.

In addition to the studies performed on mDNB, other studies were performed on NB, a structurally related compound. Previous publications have suggested that NB is a testicular toxicant (Bond *et al*, 1981), possibly with a direct effect on the Sertoli cell, however the evidence is not as substantial or as conclusive as that for mDNB. Studies conducted as part of this thesis have demonstrated for the first time the detailed histological changes which occur

within the testis and epididymis post-treatment. Alterations in the hormonal milieu post-exposure together with the demonstration of toxicity within Sertoli cell cultures is conclusive evidence that NB, like mDNB, is a Sertoli cell toxicant. Significant alterations in the levels of both serum FSH and IF inhibin were evident after treatment coupled with disruption of spermatogenesis and degeneration of germ cells over the first 7 days after exposure. Degenerating giant bodies were also observed within the epididymis of treated animals at a number of time intervals post-treatment. Studies presented in this thesis suggest that histological examination of epididymal content gives an additional index of testicular function. Further studies have also demonstrated for the first time the toxicity of NB *in vitro* using known indices of testicular function including germ cell exfoliation, morphology, lactate and pyruvate secretion and a new index of Sertoli cell function *in vitro,* basal and stimulated inhibin secretion. Significant changes in inhibin secretion were evident *in vitro* following NB administration at doses identified as causing changes in known parameters of testicular cell function in identical experiments.

In addition to causing testicular disruption a significant degree of stage synchrony was observed at the histological level after NB treatment, probably through an alteration in the duration of the spermatogenic cycle due to an arrest or increase in the duration of certain stages of spermatogenesis. Although the phenomenon of stage synchronization is not new, its induction by a testicular toxicant represents a new and potentially exciting finding which may aid in our understanding of testicular function. Induction of stage synchrony would allow the study of stage specific secretions *in vivo* in concentrations far in excess of those examined presently, making the identification and evaluation of their role in spermatogenesis easier to elucidate. In addition, seminiferous tubules isolated and cultured from these animals could aid in the investigation of selective Sertoli cell - germ cell interactions which occur in a stage-dependent manner. For a number of years research efforts have concentrated on the experimental induction of stage synchrony. To date, considerable success has been achieved using vitaimn A deficient rats. The technique is, however, extremely costly and time consuming. In future, it may be possible to administer a single or repeated oral doses of NB, induce stage synchrony and study stage specific production of paracrine mediators both *in vivo* and *in vitro*. Further work is, however, required to examine the reproducibility of stage synchronization induced by NB in individual animals in more detail, the degree of stage synchrony i.e. the percentage of tubules in one or two defined stages, and the between animal variation which may exist in both the degree of

disruption and percentage of stages synchronized. It will be particularly important to establish whether two successive doses of NB, given 1 or 2 days apart, are able to exagerrate the stage synchrony observed after a single treatment.

MAA represents an extremely useful biochemical tool with which to investigate spermatogenesis. The effects of MAA are specific and dose-related. A single oral dose results in the degeneration of pachytene spermatocytes, the number of stages in which this occurs being dependent on the dose of MAA administered. At 650 mg/kg nearly all pachytene spermatocytes except those in early- and mid- stage VII degenerate. Therefore MAA has the potential to be used to investigate Sertoli cell-germ cell interactions along similar lines to those adopted by other investigators using ethane dimethane sulphonate (EDS), the Leydig cell cytotoxin, which has been used to evaluate the concentration of intratesticular testosterone required to maintain spermatogenesis and the various roles of Leydig cells in spermatogenesis *in vivo.*. Previous experiments have examined the effect of MAA on testicular histology and temporal changes in hormone levels (Bartlett *et al*, 1988) and fertility (Ratnasooriya & Sharpe, 1989). However the effects of MAA on inhibin secretion *in vivo* were not examined. Data presented in this thesis suggests that serum FSH levels, (in most cases), correlate directly with inhibin levels in IF and presumably blood.

Previous investigators have demonstrated that some of the isomers of mNT are testicular toxicants (Ciss *et al*, 1980). Whilst overwhelming evidence suggests that DNT and TNT are testicular toxicants, the findings of this thesis suggest that mNT is not a testicular toxicant when administered in high single oral doses. From our experience, mNT has secondary effects on the testis; the primary effect of mNT at high doses, (but below the published LD_{50} level - Ciss *et al*, 1980), is the death of the animal. In chronic toxicity studies, mNT (in addition to DNT and TNT) has also been reported to induce testicular degeneration, although it is difficult to identify if this is a direct effect of the compound on the testis or a result of secondary changes in general physiology or endocrinology. Some of the isomers of mNT do, however, induce celluar exfoliation in Sertoli cell co-cultures together with morphological changes in mono- and co-culture including Sertoli cell vacuolation and significant increases in inhibin secretion. These *in vitro* results represent one of the major disadvantages with a number of indices of Sertoli cell function *in vitro*, namely that they should be evaluated in the context of *in vivo* results to confirm and investigate specific *in vivo* findings further. Previous studies have demonstrated

that germ cell exfoliation can occur following the administration of compounds and doses demonstrated to be non-toxic *in vivo*. Furthermore an essential consideration in examining *in vitro* results is to consider if toxicity observed *in vitro* is produced at relevant concentrations to those measured or predicted from pharmacokinetic studies *in vivo*. If not, then these *in vitro* studies are not representative of the *in vivo* situation. From the limited pharmacokinetic data available (Rickert, 1987) it is possible to calculate an approximate blood level (and therefore testicular level) following the administration of mNT *in vivo*. A high single oral dose (1000 mg/kg) results in a blood level which, when administered at an equivalent concentration *in vitro* induces changes in the indices of Sertoli cells function in culture typical with those observed using other known toxicants *in vitro*. These results are contrary to those found *in vivo* and again highlight the necessity to evaluate all *in vitro* data in the light of *in vivo* information.

The studies in this thesis have utilised a number of methods to evaluate spermatogenic disruption *in vivo* and *in vitro*. Of the *in vitro* techniques, primary testicular cell cultures of Sertoli cells and Sertoli cells cultured in the presence of germ cells represent techniques used successfully over the last 5 to 10 years to investigate both testicular toxicity and Sertoli cell - germ cell interactions and secretions at a physiological level. Recent advances in purifying and enriching specific complements of germ cells and adding them back to Sertoli cells in confluent monocultures have advanced our knowledge in the field of modulation of Sertoli cell secretions by certain classes of germ cells (Le Magueresse *et al*, 1986; 1988; Le Magueresse & Jegou, 1986; 1988; Pineau *et al*, 1990). From the perspective of the toxicologist these *in vitro* systems have aided the investigation of the mechanism and site of action of a number of testicular toxicants including mDNB, MAA and their metabolite(s). A number of indices of testicular function *in vitro* have been used including morphology, germ cell exfoliation and the secretion of lactate and pyruvate. Studies in this thesis have demonstrated the testicular toxicity of NB using these indices of perturbation and evaluated a new one - inhibin (discussed later).

There are numerous advantages and disadvantages to primary testicular cell culture used in the context of examining both testicular physiology (Russell & Steinberger, 1989) and identifying testicular toxicants (Gray, 1988). Further work is required using other toxicants in order to evaluate the potential of this culture system as a further avenue for investigation from both the physiological and toxicological perspective.

A major disadvantage with primary testicular cell cultures is that the epithelium must be broken down using enzymatic digestion and "re-formed" to a limited degree *in vitro*. Therefore the complex structural arrangements observed *in vivo* are not apparent *in vitro* and Sertoli cells tend to form confluent squamous monolayers which are inherently variable in response to hormones and treatments. An alternative, as described by Parvinen in 1982, is to culture seminiferous tubules isolated from adult rats. The major advantage to this system is that the structural integrity of the epithelium is maintained. A bonus is that animals may be treated with toxicants, then seminiferous tubules isolated to study known indices of testicular dysfunction *in vitro* in addition to the production of stage-specific factors by Sertoli cells and/or germ cells or paracrine factors post-exposure.

Extensive experiments generating and validating the seminiferous tubule culture system to optimise inhibin secretion have been reported in this thesis. The technique has a number of advantages over primary cell culture but requires further investigation before it can be introduced into a toxicity screening program. Clarification is required on a number of fronts. Firstly after 24, 48 and 72h in culture, are the tubules similar to those *in vivo* ? The responsiveness of the tubules in culture to hormonal stimulation remained significantly elevated at all time intervals. However in unstimulated cultures inhibin levels decreased with time in culture. This could be a sign of cellular degeneration in culture, although inhibin secretion decreases in the absence of stimulation *in vivo* and possibly this is representative of the situation observed *in vitro*. Are the tubules damaged by mechanical dissection such that intracellular enzymes are released into the medium which lyse cells ? Microscopic examination of tubules in culture suggests that, although the external integrity of the tubule is maintained, cells are exfoliated from the cut ends of the tubule. However the use of a relatively long (2cm) segment of tubule probably means that a "physiological environment" is maintained within the central section of the tubule. Does the lumen remain intact or the epithelium collapse ? Microscopic examination of tubules in culture suggests that in the central region of the tubule the lumen is visible at some stages of spermatogenesis. Preliminary histological investigations suggest that in certain sections of a cultured tubule the lumen is present. Further experiments are required though because of the difficulty in preserving these fragile pieces of tissue for histological examination, as it is impossible at present to distinguish between damage caused by poor histological technique and a poor culture system. Are the responses of the tubules in culture similar to those *in vivo* ? Data from the measurement of basal and stimulated inhibin secretion in culture

suggests that although responsiveness is maintained it is debateable if Sertoli cell bidirectional secretion occurs since if the lumen collapses, or the ends of the tubules are sealed by exfoliated cells, moieties such as inhibin probably transverse the epithelium to be secreted basally rather than apically. The ramifications for the study of other bidirectionally secreted proteins in tubule culture is unknown, but for inhibin results suggest that it makes little difference.

Taking into account the as yet unknown factors outlined above, the culturing of seminiferous tubules represents a step forward in our ability to study the control of seminiferous tubule physiology. Previous investigators have demonstrated in primary culture that Sertoli cell secretions are modulated by specific complements of germ cells. Exciting new evidence using this technique suggests not only that elongate spermatids control inhibin secretion but that effects induced by MAA during the first 3 days *in vivo* (in terms of inhibin secretion), can be mimicked *in vitro* and possibly suggest that pachytene spermatocytes may also modulate inhibin secretion. This technique has further applications: It could be used to study the possible modulation of Sertoli cell secretions such as transferrin, ceruloplasmin, ABP, SCP-2 by germ cells as well as the numerous other proteins with as yet unknown functions. An elaboration of the technique would be to dissect and culture specific stages of spermatogenesis using trans-illuminated-assisted micro-dissection (Parvinen, 1982) which would allow the investigation of proteins secreted by Sertoli cells in a stage specific manner, utilising 2-dimensional gel electrophoresis to study the secretion of Sertoli cell proteins either stage dependently or in the absence of certain germ cell classes (induced by MAA *in vivo*) or by combining the two techniques. A major application of this would be to study the stage-specificity of the early effects of various testicular toxicants, as these clearly cause stage-related disruption, as has been demonstrated in this thesis. The major disadvantage to the dissection of lengths of seminiferous tubules in specific stages of spermatogenesis is that it is time-consuming and difficult since some stages are very small in length. Further experiments could include studying paracrine communication between Sertoli cells, Leydig cells and germ cells. Recent advances using the Leydig cell cytotoxin EDS suggest that Sertoli cells and/or germ cells may be involved in the initiation of differentiation and development of Leydig cells, possibly as a result of damage to the seminiferous epithelium caused by androgen deprivation. It is possible that, as a result of testosterone withdrawal, germ cells degenerate and Sertoli cells release a factor(s) which stimulates Leydig cell differentiation as a physiological response to the lack of androgens. This factor(s) could be isolated in cultures of

seminiferous tubules dissected following Leydig cell removal *in vivo*. While much of the above is educated conjecture at present, the technique of culturing seminiferous tubules opens new avenues into the investigation of spermatogenesis and its paracrine control.

NB has been demonstrated in these studies to induce stage synchrony, therefore NB is of potential use in the study of stage-dependent changes in Sertoli cell secretory function *in vivo*. Inducing stage synchrony in the testis presents an opportunity to isolate IF, seminiferous tubule fluid or testicular homogenates during a time when it would be possible to accurately predict stages of spermatogenesis within the whole testis. These fluids could then be analysed for paracrine mediators involved in spermatogenesis which are produced specifically during certain stages. Advancing this idea a step further and combining it with the isolation and culture of seminiferous tubules, it would be possible to isolate with ease lengths of seminiferous tubules in stages which are normally very small in length and difficult to dissect. Similarly the administration of toxicants such as MAA would allow the investigation of germ cell modulation of Sertoli cell secretion in a stage-dependent manner. In addition, the role(s) of the Leydig cell and paracrine mediators secreted between Leydig cells, Sertoli cells and germ cells could be investigated. Alternatively EDS could be used to remove Leydig cells *in vivo* to study their regeneration - do they regenerate at a faster rate around stage VII tubules *in vivo* -"the androgen dependent stage" (Sharpe *et al*, 1990) or at earlier stages? Do Leydig cells regenerate around stage VII tubules in greater numbers than at other stages *in vivo* ? The scope for potential investigation is great.

Primary cultures of Sertoli cells and Sertoli cells in the presence of germ cells are very useful as models for the study of mechanisms of toxicant-induced perturbation. If the site of action of the toxicant is the Sertoli and/or germ cells then the culture system is useful to study parameters of cellular function and the generation of toxic metabolites in culture. Foster, (1989) demonstrated that the effects of mDNB were the result of metabolites of mDNB formed in culture. These reactive species (including free radicals) induced toxicity and their effects could be negated or enhanced by the addition of certain compounds to the cultures, (cysteamine - a free radical scavenger, and diethylmaleate - which depletes glutathione levels repectively), (Foster, 1989). Similarly in the present studies, NB has been shown to produce similar effects in culture to those of mDNB (Foster *et al*, 1987) and further work may evaluate the mechanism of toxicity of NB in primary culture - is it similar to that of mDNB ? These *in vitro* techniques used for the identification of testicular

toxicants will never replace *in vivo* methods but in collaboration may aid in our understanding of the complex processes involved in spermatogenesis and the interactions of toxicants which disturb these processes.

Studies in this thesis have demonstrated that inhibin represents a marker of early toxicant action on the Sertoli cell, however further work is required to evaluate its sensitivity. FSH is another peripheral marker of testicular dysfunction but it is insensitive to small changes in the testis and requires massive germ cell degeneration before significant rises occur. Contrary to what was predicted, experiments performed *in vivo* suggest that when testicular dysfunction is induced by a toxicant, i.e. NB or MAA, serum FSH and IF inhibin levels rise in parallel with one another. However following mDNB administration inhibin levels were significantly elevated 24h after exposure while FSH levels did not increase significantly until 3 to 7 days post-treatment. This would tend to suggest that inhibin may be a more sensitve indicator of testicular dysfunction than FSH. Unfortunately levels of inhibin in peripheral serum were not measured in these experiments because they were extremely variable between assays for the same sample and between experiments. In addition, results in the RIA did not agree with those obtained in the sheep pituitary bioassay for the same sample. This was probably due to cross reactivity within the RIA of coagulation factors in blood since inhibin levels measured in plasma were repeatable and in agreement with bioassay data. In retrospect, plasma samples should have been collected from animals post-treatment in order to evaluate inhibin levels after exposure to toxicants. The MAA experiment has been repeated and results for plasma inhibin levels should soon become available.

In primary culture inhibin measurement represents another index of Sertoli cell function. In parallel with changes in other known indices of Sertoli cell function *in vitro*, inhibin levels also changed in the presence of toxicants in a dose-dependent manner. In the presence of increasing concentrations of mDNB inhibin levels increased dose-dependently except at a dose of $10^{-4}M$. The response of inhibin to NB administration was also dose-dependent but followed a biphasic pattern. Following administration of the isomers of mNT to Sertoli cell cultures inhibin levels also increased, again in a dose-dependent manner. In general these patterns of response in terms of inhibin secretion did not vary between Sertoli cell mono- and co-cultures. However the magnitude of response to toxicants was significantly greater in the presence of germ cells. Indeed co-cultures secreted more inhibin at a basal and stimulated level than did Sertoli cells alone, suggesting that germ cells modulate Sertoli cell

inhibin secretion in the presence of toxicants, but in the absence of germ cells the pattern of response of the Sertoli cell to toxicants did not vary. Therefore the measurement of inhibin secretion in primary co-culture may in some cases represent a sensitive index of testicular cell perturbation *in vitro*, however further work is required to clarify these findings, particularly the biphasic results recorded in the presence of NB.

The measurement of inhibin in seminiferous tubule cultures has demonstrated that elongate spermatids modulate Sertoli cell inhibin secretion. In addition, the effects of MAA on IF levels of inhibin evident *in vivo* at 1 and 3 days post-treatment were also observed as an increase in the secretion of basal and stimulated inhibin secretion in culture. This suggests that the culture system is representative of events *in vivo* post-treatment. However it must be remembered that the levels of inhibin measured *in vitro* are probably a reflection of basal and apical secretion. Since Maddocks & Sharpe (1989) have demonstrated that in the adult rat inhibin enters the systemic circulation via apical secretion followed by resorption from the rete testis, basal secretion into IF may not be as important in endocrine terms. Therefore the measurement of peripheral inhibin levels following MAA administration is required in order to piece together the full picture of *in vivo* and *in vitro* results. Furthermore Risbridger *et al* (1989) have demonstrated that Leydig cells secrete inhibin. While the physiological relevance of this is unknown, further experimental work by Sharpe & Maddocks (1989) has demonstrated that the Leydig cell contribution to the testicular and blood levels of inhibin is insignificant. Although this must be considered in future if inhibin becomes an index of testicular function, the contribution of Leydig cells is significantly less important than that made by the Sertoli cell.

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