THE PHYSIOLOGY AND IMMUNOLOGY OF THE ENDOCRINE TESTIS

by

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Knowledge and wisdom far from being one,
Have oft times no connexion. Knowledge dwells
In heads replete with thoughts of other men;
Wisdom in minds attentive to their own.
Knowledge, a rude unprofitable mass,
The mere materials with which wisdom builds,
Till smooth'd and squar'd and fitted to its place,
Does but encumber whom it seems t'enrich.
Knowledge is proud that he has learn'd so much;
Wisdom is humble that he knows no more.

William Cowper (1731-1800)
Cowper Poetical Works

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ABSTRACT

The testis has long enjoyed a reputation as an immunologically privileged site. This thesis re-examines the immune privileged status of the testis, and likely mechanisms involved in providing this privileged status.

The privileged status of the rodent testis was confirmed with thyroid and pituitary allografts. The effects of cryptorchidism, efferent duct ligation, and heat on the testis did not affect intratesticular graft survival. Increased lymph flow after hCG administration was also unable to initiate an immune response. Specific destruction of Leydig cells by EDS prior to transplantation led to rejection of thyroid allografts, suggesting that Leydig cells may be involved in immunoprotection in the rat testis.

To investigate the role of lymph in conveying graft-derived hormones and possible immunosuppressive factors from the testis, experiments were performed in sheep - a species in which testicular lymphatics are easily accessed. All previous studies on intratesticular graft survival have been performed in rodents and related species. This study also provided an opportunity to investigate the immune status of the testis in a species not related to rodents. While intratesticular thyroid autografts survived and concentrated iodine four weeks after transplantation, thyroid and pituitary allografts were promptly rejected in this time. None of the grafts were able to secrete detectable levels of hormones, or respond to other hormonal stimuli. These studies suggested that the immune

state of the ovine testis was superior to that of the rodent testis and that the immune privileged status of the rodent testis was not necessarily a general mammalian characteristic.

To allow comparison of the interstitial environment of these two species, interstitial fluid was sampled. A number of authors have suggested that the high levels of intratesticular steroids are responsible for the immune privilege afforded this organ in rodents. Interstitial fluid in rodents has been collected by a method termed Drip-collection, although the physiological state of such fluid is open to question. This technique has many shortcomings so an alternative method was developed using the Push-pull cannula often employed in neurophysiological studies. Fluid collected with this cannula was different in compositition to that collected by drip collection. Of most consequence for the studies of this thesis was the indication that intratesticular steroid levels are no greater than testicular venous blood levels, suggesting that previous values possibly over-estimated actual concentrations by up to ten times. The push-pull technique was also used to monitor the composition of interstitial fluid after a variety of experimental manipulations, including heat treatment, efferent duct ligation, hCG injection, and hypophysectomy with pregnenolone supplementation. The results of these studies suggested that Leydig cells secrete as much testosterone into blood as they do into interstitial fluid, and that levels of testosterone in interstitial fluid may arise more as a consequence of blood levels, and are regulated by changes in blood flow and vascular exchange. A small study on rams and boars found similar levels of testosterone in interstitial fluid to those found in the rat, and no other reports suggest that rams and rats have different steroid

profiles in any other respects.

These studies suggested that the immune privileged status of the rodent testis was unlikely to be maintained by steroids. At this stage it was decided to examine the role of specific cell types within the testicular interstitium. During a visit to Finland, a co-operative study was initiated with Prof. M. Niemi and Dr P. Pollanen in Turku, to examine the immunological cells of the rat and ram testis. This study demonstrated similar lymphocyte populations in both species. While the rat possessed numerous macrophages in the interstitial region, the ram was almost lacking in these cells. Such a variance could account for the difference in the immune state of the testes of these two species.

The use of the testis as a site for endocrine transplants remains an exciting prospect. The work in this thesis provides a new approach for work in this field with the indication that the immunologically privileged status of the rodent testis may not be a general mammalian characteristic. Some new concepts on the possible mechanisms that afford an immunologically protected environment in the rodent testis are presented.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Simon Maddocks.

ACKNOWLEDGEMENTS

It is with great pleasure that I acknowledge the assistance of all those who helped me with the studies presented in this thesis.

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Professor Mikko Niemi and Dr Pasi Pollanen of the Institute of Biomedicine, Turku, Finland made available their laboratory facilities and excellant hospitality during a short visit to Finland. The investigations on immunocompetant cells of the testis were initiated during this visit, but were hampered by industrial action in the country at the time. That Prof. Niemi and Dr Pollanen were able to complete much of the work after my return to Australia is sincerely appreciated. I am also grateful to them for allowing me to include the work in this thesis.

I wish to acknowledge the expert assistance of the staff of the farm and the engineering workshops at the Waite Institute. I am also indebted to the librarians at the Institute for their untiring efforts in obtaining rare and at times obscure, manuscripts. Ms M. Morris of the Biometry section gave invaluable assistance with the statistical assessment of results.

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Finally, I am eternally grateful for the love and support of my wife Vicky and daughter Carolyn. They went without so much, while I attended to my studies and the writing of this thesis. Thanks also to my parents Ian and Diana for their continued support and encouragement.

PREFACE

Aspects of the work presented in this thesis have been reported elsewhere:

Abstracts

Maddocks, S; Cormack, J and Setchell, BP (1985)
The failure of thyroid allografts in the ovine testis.
Proc. Aust. Soc. Reprod. Biol. 17 : 51.

Maddocks, S and Setchell, BP (1986)
The composition of the interstitial fluid of the rat testis.
IV European Testis Workshop Miniposters: 154.

Maddocks, S; Zupp, JL; Sowerbutts, S; and Setchell, BP(1986)
Testosterone levels in rat testicular interstitial fluid.
Proc. Aust. Soc. Reprod. Biol. 18 : 58.

Pollanen, P and Maddocks, S (1987)
Macrophages, lymphocytes and MHC II antigen in the ram and the rat testis.
VII Wkshop. on Development and Function of the Reproductive Organs: in press.

Papers

Maddocks, S; Sowerbutts, SF and Setchell, BP (1987)
The effects of repeated injections of human chorionic gonadotrophin on vascular permeability, extracellular fluid volume and the flow of lymph in the testes of rats.
Int. J. Androl., in press.

CHAPTER 1: INTRODUCTION

1.1. INTRODUCTION

The testis, like the brain, anterior chamber of the eye, and the hamster cheek pouch, is often cited as an immunologically privileged site (Barker and Billingham, 1977). It protects tissue grafts from the immunological rejection that they would otherwise suffer in other regions of the body. Such grafts are placed into the interstitial region of the testis, and a number of authors have attempted to explain the immunologically privileged status of this region by factors present in the interstitial milieau.

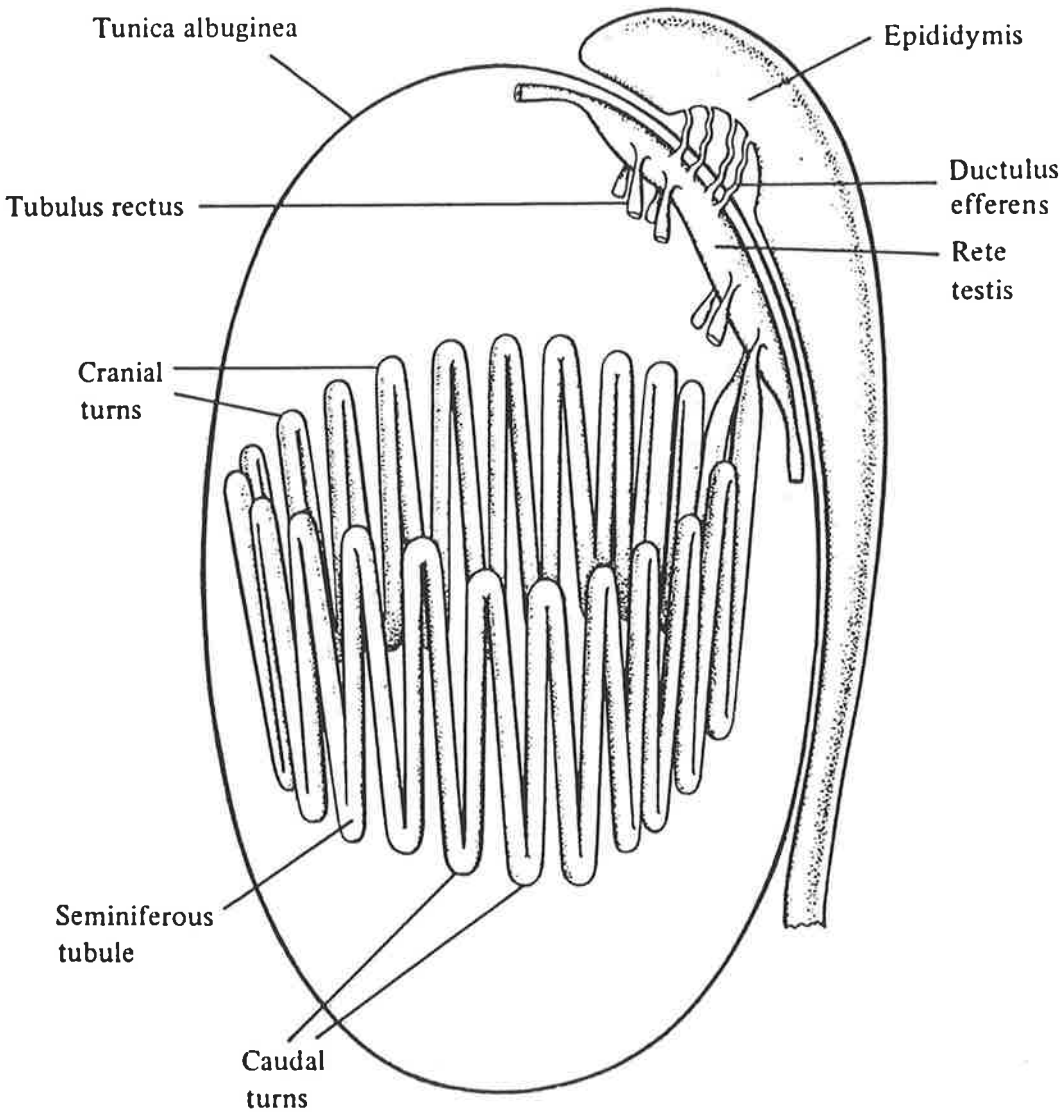
The work in this thesis re-examines the immunologically privileged status of the testis. Both the physiology and the immunology of the testicular interstitium may contribute to the immune privileged status afforded this region, and the following review introduces these two aspects of the endocrine testis.

1.2. THE PHYSIOLOGY OF THE ENDOCRINE TESTIS

1.2.1. Introduction

The testis is an extremely complex organ producing the male gametes (spermatozoa) and the male sex hormones (androgens). In 1668 Regnier de Graaf showed convincingly that the testis consists of a series of elongated, convoluted tubules which produce semen. These tubules are usually two ended (Clermont and Huckins, 1961) and open at both ends into the rete-testis (Roosen-Runge, 1961) (see Figure 1.1.). The production of spermatozoa and their successful delivery from the

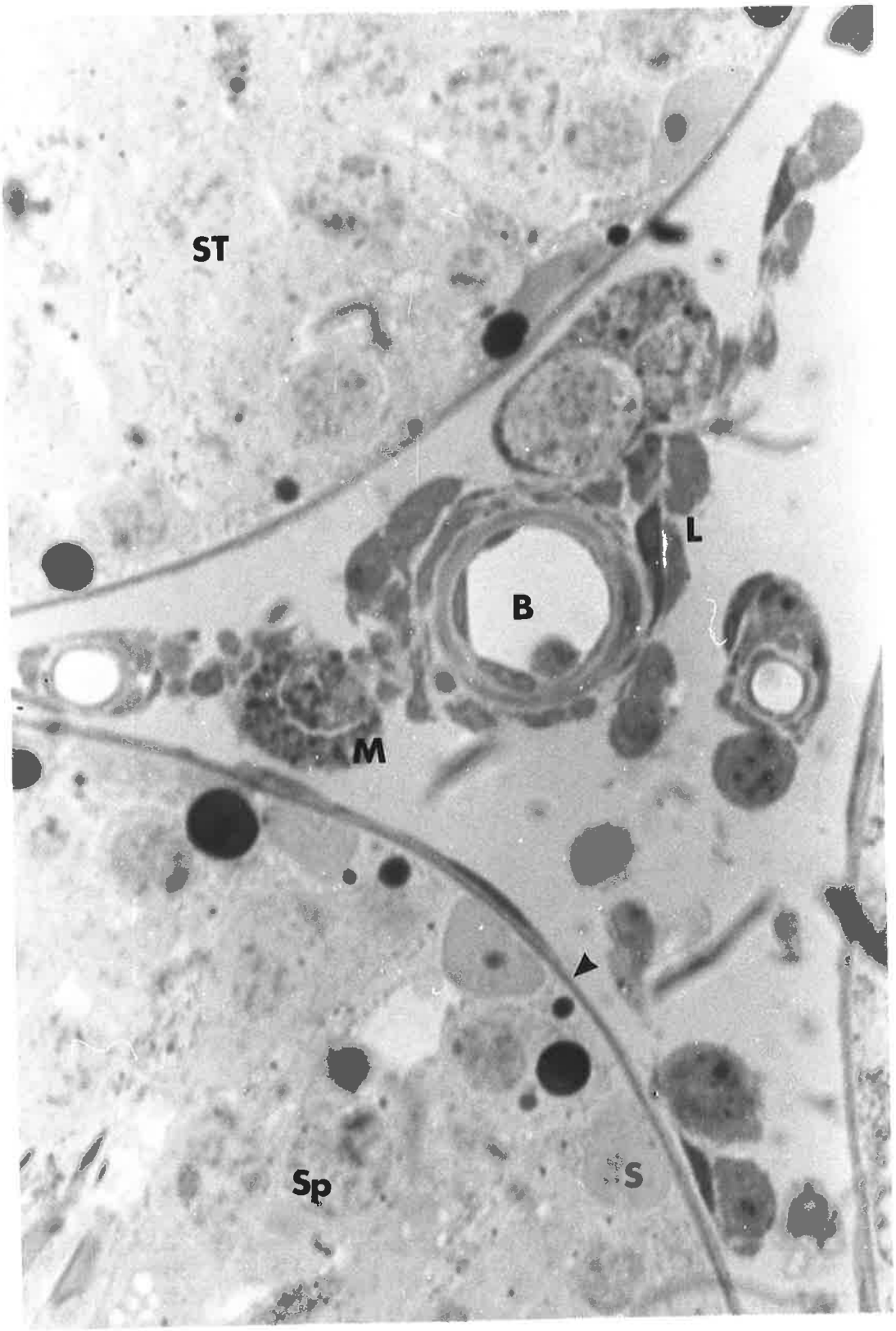
Figure 1.1. Diagram showing the arrangement of one of the seminiferous tubules and the rete testis in the testis of a rat (from Clermont and Huckins, 1961)


Tunica albuginea
Epididymis
Ductulus efferens
Tubulus rectus
Rete testis
Cranial turns
Seminiferous tubule
Caudal turns

male reproductive tract is largely dependent upon an adequate supply of androgen - an endocrine function of the testis. This was demonstrated in 1849 by Berthold in one of the first experiments in endocrinology when he showed that regression of the secondary sexual characteristics in a castrated rooster could be prevented by ectopically re-implanting part of the testis. Because the seminiferous tubules are cylindrical structures, an interstitial space is formed between adjacent tubules (Figure 1.2.). Blood vessels, lymphatics and nerves, which do not penetrate the seminiferous tubules, along with the hormone-producing Leydig cells are usually found in this extracellular region. However, the specific composition and anatomy of this extracellular region does vary between species (see Fawcett et al, 1973). The whole structure is encased in a tough capsule.

The boundary wall of the seminiferous tubules consists mainly of myoid cells and non-cellular material, involved in movement of the tubules. They are closely associated with, and probably influence the Sertoli cells which extend from the boundary wall to the lumen of the tubule. The Sertoli cells have contact with all other cell types in the epithelium and the various germ cells are either sandwiched between adjacent Sertoli cells or found embedded in the luminal surface of the Sertoli cell cytoplasm. Germ cell development is influenced by the pituitary gonadotrophin FSH, and the testicular androgen testosterone, both of which appear to act through the Sertoli cells. The Sertoli cells secrete most if not all of the fluid found within the tubular lumen (Setchell, 1969), and can do so against considerable hydrostatic and diffusional gradients. Part of this secretion is now recognised as being hormonal and the protein hormone Inhibin has received much attention as a Sertoli cell product capable

Figure 1.2. Cross-section of a testis from a mature rat. The interstitial space between the seminiferous tubules (ST) contains the Leydig cells (L), macrophages (M) and blood vessels (B) and is filled with extracellular interstitial fluid. The outer layer of the seminiferous tubules (▶) consists of a layer of myoid cells surrounding an inner layer of epithelial cells. The inside of the tubules is lined by Sertoli cells (S) which enclose the spermatogenic cells (Sp). Light micrograph X 1250.


ST
L
B
M
Sp
S

of regulating pituitary FSH release. The boundary wall of the tubules physiologically separates the luminal compartment from the interstitial tissue by cellular barriers that affect the free exchange of water-soluble materials, and as a consequence luminal fluid differs markedly in composition from interstitial fluid.

The Leydig cells of the interstitium are responsible for androgen production and this process is influenced by the pituitary gonadotrophin LH. Recent evidence suggests local regulation may also occur involving factors present in testicular interstitial fluid (Sharpe and Cooper, 1984; Rommerts et al, 1986), possibly derived from Sertoli cells (Benahmed et al, 1985a).

In the last ten years there has been a growing interest in other cell types of the interstitium, and macrophages, fibroblasts, endothelial cells, mesenchymal cells and mast cells have been described. The importance of many of these other cell types and their role in the testis is yet to be fully realised, although it is likely that they are involved in the endocrine activities of this organ. Macrophages are frequently found in close association with Leydig cells (Connell and Christensen, 1975; Wing and Lin, 1977; Miller et al, 1983; Niemi et al, 1986) and morphological changes in Leydig cells (caused by cryptorchidism, LH withdrawal, seasonal dysfunction) are concomitant with morphological changes in the macrophages (see Bergh, 1985; Gondos et al, 1980; Wing and Lin, 1977). The endothelial cells line the blood vessels and the lymphatics, and together with mesenchymal cells and macrophages have been considered as possible Leydig cell precursors.

There remains much uncertainty as to the other functions of these cells however. Some of them (eg. macrophages) have traditionally been

recognised as important components of the immune system and the immunology of the interstitial region will be discussed in later sections of this review (see 1.3.).

1.2.2. The Leydig cells

In 1850 Franz Leydig described the presence of interstitial cells situated between the seminiferous tubules in the testis, but did not suggest any particular function for them. These cells were named after Leydig by later workers and in 1903 Bouin and Ancel provided circumstantial evidence that they were the source of the male hormone. However this proposal remained controversial until the late 1920's, and some workers continued to suggest that the Leydig cells modified and stored hormones produced in the tubules (see Setchell, 1984). In 1965, Christensen and Mason demonstrated directly that the interstitial tissue produced much more testosterone than the seminiferous tubules. Bell et al (1971) showed that the tubules could convert labelled steroid precursors into other steroids, however the interstitial region, and particularly the Leydig cells seemed to be the only cells in the testis able to synthesize androgenic steroids from cholesterol or acetate.

1.2.2.1. Anatomy

The mammalian Leydig cell is a relatively large, polyhedral, and epithelioid cell. It is surrounded by a typical plasma membrane which is frequently folded with microvilli. It usually has a single, eccentrically located nucleus, although binucleated cells are not

uncommon. The nucleus is spherical or ovoid and distinctly vesicular, and contains one to three large nucleoli. The remaining chromatin is present as granules distributed predominantly around the nuclear membrane giving this membrane an appearence of exaggerated thickness when seen with the light microscope (Hooker, 1970). No other cell of the interstitial tissue has a nucleus with these characteristics, making identification relatively easy. The cytoplasm is usually abundant, with a prominent amount of smooth endoplasmic reticulum. This is particularly so in guinea pigs, opossums, mice and boars. There are also scattered patches of rough endoplasmic reticulum which interconnect with the smooth ER. The mitochondria are of moderate size and number and appear in the characteristic lamellar form, although many are tubular (Christensen, 1975). Tubular cristae are not particularly distinct in the human, guinea pig or rat. The Golgi complex is well developed and often found at one pole of the nucleus. The cytoplasm of the mature cell contains lipid globules of different size and number depending on the species. They are particularly abundant in the cat, and common in humans, guinea pigs and mice. These globules were mentioned in Leydigs' original description and Loisel (1903) guessed that the secretion of these cells may be lipid in nature. Reinke crystals, microtubules, and microfilaments are also found in the cytoplasm along with primary lysosomes, digestive vacuoles (secondary lysosomes) and residual bodies (late secondary lysosomes). These residual bodies often take the form of lipofuscin granules when lipid droplets are present, but are usually rare in species in which the cells lack lipid droplets such as rat or opossum. Boar Leydig cells do not show lipid droplets, however they do possess residual bodies containing an unusual reddish pigment. Leydig cells

range in size from about 10 um diameter in the human to 30 um in the boar, and constitute anything from 2% (rat) to 37% (boar) of the interstitial volume. The number of Leydig cells in the boar is extreme and they virtually fill the interstitial tissue.

Most seasonal breeders also show Leydig cells of differing appearance depending on the season. In such animals, Leydig cells appear to transform from an "undifferentiated" interstitial cell just before the breeding season, into an active Leydig cell with abundant smooth ER, possessing mitochondria with tubular cristae, and possibly a number of medium sized lipid droplets. Following the period of activity, they regress, and lose the specific Leydig cell characteristics (Wing and Lin, 1977; Hochereau de Reviers and Lincoln, 1978).

Two generations of Leydig cells have been described for most mammals studied, although three populations have been found in the human (Mancini et al, 1965; Pelliniemi and Niemi, 1969) and pig (van Straaten and Wensing, 1978; van Vorstenbosch et al, 1984). The first is termed the "fetal" generation because this is when they first appear. Their function is to secrete the androgens responsible for differentiation of the male reproductive tract but not the regression of the paramesonephric Muellerian ducts. They function independently of gonadotrophins and regress at birth or in the early post-natal period, the extent of regression depending on the species. Within a few months after birth, the interstitial tissue of the human testis becomes essentially devoid of Leydig cells and remains in this condition throughout childhood until puberty. On the other hand, there is apparently little involution postnatally in the guinea pig or in the mouse. The rat undergoes some decrease in Leydig cell number

postnatally, however there remains a population of Leydig cells which continue to secrete low, but measurable amounts of testosterone in plasma. This apparently initiates neural changes important for subsequent development of male behaviour. In the human and the pig, a second population of Leydig cells has been described which are gonadotrophin dependent, transient, and present in the perinatal period. The final generation in all species appear to be initiated post-natally at the time of puberty. They differentiate from interstitial cells to aquire the characterstics of the "adult" Leydig cell. Exactly which cells are the precursors for this population has yet to be determined. Fibroblast-like or mesenchymal cells (Mancini et al, 1965; Kerr and Sharpe, 1985), macrophages (Clegg and MacMillan, 1965a), and endothelial cells (Laws, 1985) have been suggested as Leydig cell precursors, as have dedifferentiated fetal Leydig cells remaining in the testis (Prince, 1984). Proliferation of those organelles involved in manufacturing secretory products takes place (mitochondria, smooth ER, Golgi apparatus) with nuclear changes and an increase in the microvilli processes on the cell surface (see Burgos et al, 1970; Christensen, 1975). During maturation, the number of Leydig cells and their size increases (Knorr et al, 1970; Tapanainen et al, 1984) along with LH receptor numbers (Ketelslegers et al, 1978). Mitosis of fully differentiated Leydig cells may also occur to a lesser extent and Christensen and Peacock (1980) and Amat et al (1986) have shown that Leydig cell division and differentiation is still possible in mature rats.

In most species Leydig cells occur as clusters in the triangular intertubular space, and as strands between closely opposed tubules (see Burgos et al, 1970; Fawcett et al, 1970; Fawcett et al, 1973;

Christensen, 1975; Neaves, 1975). In the sexually mature animal, the plasma membrane of the Leydig cell has many specialisations including junctional complexes, projections and surface indentations that are of both the smooth and coated variety (Connell and Christensen, 1975). Surrounding clusters of Leydig cells is a basal lamina of variable width and disposition that appears to function as a supporting structure in conjunction with collagen fibres located around the Leydig cells and basal lamina. These collagen fibres are few in the rat and are usually found in bundles of several fibres, which loop out over the Leydig cells and extend into the fluid space (Clark, 1975).

Much of the available information on Leydig cell function and metabolism has been derived from in vitro studies using isolated cells in culture. Because the interstitial tissue of the laboratory rat is scarcely attatched to the seminiferous tubules, its isolation in the past was accomplished by simply grasping the tubules in a piece of testicular tissue and pulling them out. When the tubules were removed the web of interstitial tissue remained (Christensen and Mason, 1965). More recently techniques have been developed where the decapsulated testis is incubated with digestive enzymes such as collagenase to assist in the separation of tubules (Payne et al, 1982; Shaw et al, 1979). The population of interstitial cells obtained in this manner can then be further purified by passing the cells down a Percoll gradient (Browning et al, 1981). These procedures yield a reasonably pure population of Leydig cells for in vitro studies. However, such studies remain limited temporally to relatively short intervals, despite the use of carefully regulated culture systems (see Browning et al, 1983). Molenaar et al (1983) examined three different isolation procedures used to obtain Leydig cells and found the steroidogenic

activity of isolated cells varied between different methods of isolation. This seemed partly attributable to the amount of cell damage caused during isolation. Aquilano and Dufau (1984) have recently used a combination of centrifugal elutriation and centrifugation on Metrizamide gradients to procure reportedly 100% pure Leydig cell preparations.

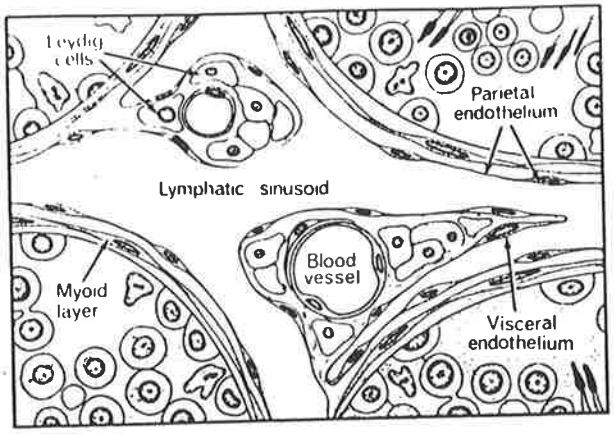
Isolation studies have led to reports of heterogeneity of isolated Leydig cells (Payne et al, 1980; Chen et al, 1981; Cooke et al, 1981) with two populations being found in the adult rat testis and classified as LH responsive or LH unresponsive (see Sharpe, 1982). However, this subject remains controversial with Aquilano and Dufau (1984) reporting the active Leydig cell population to functionally consist of only one population of Leydig cells with comparable LH receptor numbers, steroidogenic activity and susceptibility to gonadotrophic desensitization.

1.2.2.2. Relationship to blood vessels, lymph vessels and seminiferous tubules

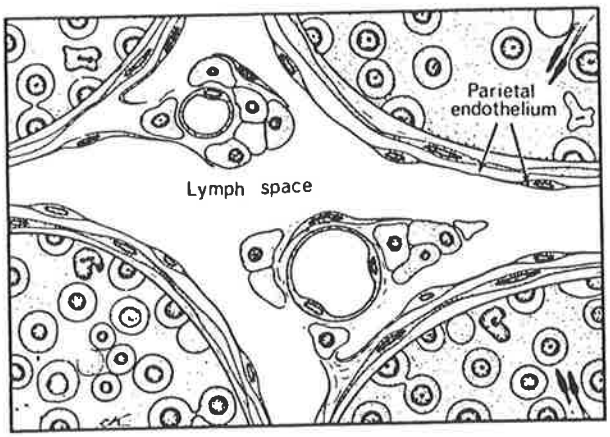
The relationship between the walls of the tubules, the Leydig cells, and the blood and lymph vessels varies amongst different species. In 1973, Fawcett et al studied the intertubular lymphatics in 14 species. The abundance of Leydig cells, the amount of intertubular connective tissue and the location and degree of development of the lymphatics allowed these authors to define three main categories of interstitial tissue organisation. The following descriptions, and Figure 1.3., are from Fawcett (1973) who increased this classification to four groups. The first group (Figure 1.3.a) is characterised by the

Figure 1.3. Diagram showing the variation in the anatomy of the interstitial tissue in several species. (a) Guinea Pig, showing Leydig cells clustered around blood vessels, with the whole groups of cells completely surrounded by endothelial cells and floating in a lymphatic sinusoid, the contents of which also bathe the walls of the seminiferous tubules. (b) Rat, similar to the Guinea Pig except that the groups of Leydig cells are surrounded by an incomplete layer of endothelial cells. (c) Ram, showing the Leydig cells either in groups near a capillary or in separate clusters embedded in loose connective tissue which also contains lymph vessels and other blood vessels. (d) Boar, showing the interstitial space crammed with closely packed Leydig cells with a few small blood and lymph vessels (from Fawcett, 1973).

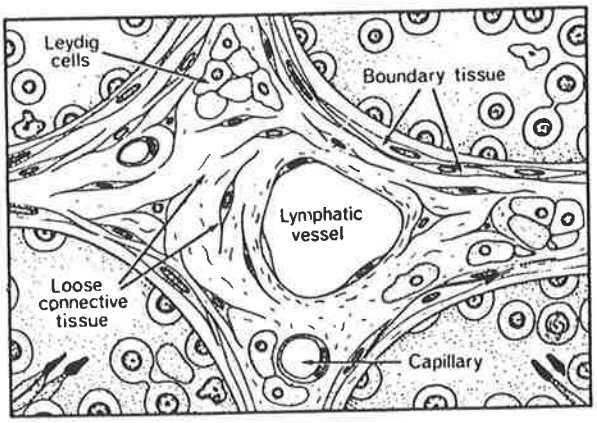
a.


Leydig cells
Parietal endothelium
Lymphatic sinusoid
Blood vessel
Myoid layer
Visceral endothelium

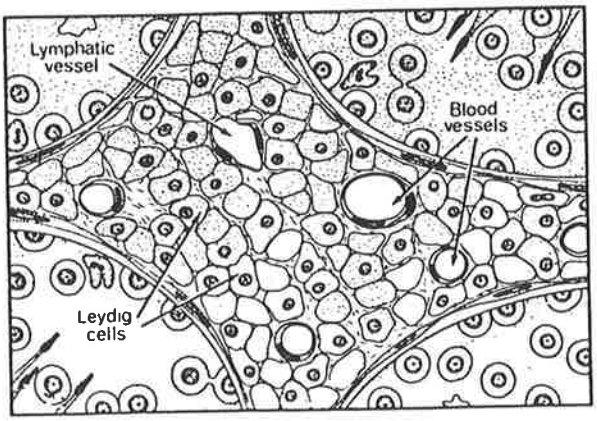
b.


Parietal endothelium
Lymph space

c.


Leydig cells
Boundary tissue
Lymphatic vessel
Loose connective tissue
Capillary

d.


Lymphatic vessel
Blood vessels
Leydig cells

guinea pig, in which the Leydig cells comprise a small fraction of the testicular volume, and occur in clusters closely applied to blood vessels. The greater part of the interstitium is occupied by extensive lymphatic sinusoids of irregular outline. These are bounded by a "visceral" (or interstitial; Clark, 1975) layer of attenuated endothelium covering the vessels and their associated Leydig cell clusters, and a "parietal" (or peritubular; Clark, 1975) layer of endothelium closely applied to the myoid layer of the seminiferous tubules. The endothelium of the lymphatic sinusoids in this space is generally continuous. Narrow sheets of collagen bounded by two layers of endothelium attach the vascular-endocrine complexes to the tubules. In this species therefore, Leydig cells are interposed between the walls of the blood vessels and the endothelium of the lymphatic sinusoids.

In the second group (Figure 1.3.b), characterised by the rat and mouse, interstitial organisation is basically the same as in the first group. However the visceral layer of endothelium is discontinuous over wide areas so that Leydig cells are directly bathed in lymph.

The majority of larger mammals, including the bull, ram, elephant, monkey and man, have a very different interstitial organisation (Figure 1.3.c). In these species, Leydig cells do not have such an obvious association with blood vessels, occuring in clusters of varying size, scattered in an oedematous loose connective tissue which is drained by conspicuous lymph vessels located centrally or eccentrically in each intertubular area.

The fourth group (Figure 1.3.d) includes the domestic boar, warthog, zebra and naked mole rat. Closely packed Leydig cells occupy large intertubular spaces and comprise up to 50% of the volume of the

testis. There is very little interstitial connective tissue in these species, and small lymphatic vessels are infrequently encountered.

Given that the Leydig cell is an important source of androgen in the testis, these species differences in interstitial organisation have obvious implications for how androgen is partitioned between the vascular and intratesticular circulation. Fawcett et al (1973) hypothesized that in the rodents (Groups 1 and 2), movement of testosterone from the Leydig cells could be envisioned as a release of androgens into the blood capillaries and into the protein-rich extracellular fluids that move from the blood vascular system into the lymphatic sinusoids surrounding the seminiferous tubules. These authors suggested that the lymphatic route of testosterone transport might be the primary source of androgens for the seminiferous tubules in these animals. In the larger mammals of Group 3, blood capillaries are in much closer contact with the tubules and therefore may directly supply testoterone to the tubules. Lymphatics in these species are believed to be more involved in return of extracellular fluid to the general circulation, than in distribution of testosterone. The large mass of Leydig cells in Group 4 is probably able to directly provide testosterone to the tubules without the need for any indirect system such as the lymphatics or blood vascular system (Connell and Connell, 1977). The high density of Leydig cells in these species probably relates to the production of other hormones such as oestrogens and pheromones (eg. "boar taint", 5α-androst-16-en-3-one).

1.2.2.3. Relationship to other cells in interstitial tissue

While the Leydig cell is widely recognised as the major cell type

of the interstitial compartment of the mature testis (Niemi and Ikonen, 1973), a number of other connective tissue cells have been reported in this region including fibroblasts, macrophages, mast cells and lymphocytes (Christensen, 1975). Ewing et al (1979) and Niemi et al (1986) have reported that testicular macrophages in the rat may constitute up to 25% of interstitial cells and Bergh (1985) estimated one macrophage per four Leydig cells. Macrophages have frequently been found in close association with Leydig cells (Connell and Christensen, 1975; Wing and Lin, 1977; Miller et al, 1983; Niemi et al, 1986) and morphological changes in Leydig cells (caused by gonadotrophin withdrawl, cryptorchidism or seasonal dysfunction) are concommitant with morphological changes in the macrophages (see Wing and Lin,1977; Gondos et al, 1980; Bergh, 1985). The importance of this association is unclear although Bergh (1985) indicated that the morphology of the two cell types is correlated such that a functional inter-relationship is likely. Indeed, macrophage association with Leydig cells leads to considerable contamination of Leydig cell preparations, and similarities in function between the two cell types has led Niemi et al (1986) to suggest that this may in fact account for "unwarranted" suggestions of Leydig cell heterogeneity (see Cooke et al, 1981). Milewich et al (1982) found peritoneal macrophages to be steroidogenic, and Molenaar et al (1984) have shown macrophages to possess common surface antigens with Leydig cells. Wahlstrom et al (1983) and Hovatta et al (1986) have found macrophages which contain FSH demonstrable by immunohistochemistry in the human testis , suggesting that these cells may also respond to gonadotrophins. Miller et al (1983) found that portions of Leydig cells may be endocytosed by macrophages, supporting the suggestion that testicular macrophages are

involved in Leydig cell function. Yee and Hutson (1985) demonstrated that conditioned medium from cultures of testicular macrophages stimulated testosterone production when added to Leydig cells in vitro. On the other hand, Niemi et al (1986) observed macrophages in prepubertal animals which may imply other functions such as immunological roles, for these macrophages. Most of these studies have been carried out with rats and the situation in other species is yet to be fully investigated.

Leydig cells have been found in the boundary tissue of the seminiferous tubules in guinea pigs (Fawcett et al, 1970) and man (Fawcett and Burgos, 1960) in association with the myoid cells and fibroblasts of this tissue. These Leydig cells may be important in Leydig cell - Sertoli cell interactions and Bergh (1982,1983) has shown that peritubular Leydig cells in the rat testis change in size according to the stage of the spermatogenic cycle in the adjacent seminiferous tubule. Macrophages have also been reported in this region of the rat testis (Ross, 1967). This peritubular region is closely bounded by the peritubular or parietal endothelium, and Leydig cells of the interstitium which are not necessarily incorporated in the boundary tissue, but which lie adjacent to the seminiferous tubules are in close association with this endothelium (Clark, 1975). This may be of consequence in affecting distribution of hormones produced by these cells. It has also been suggested that newly differentiated Leydig cells may arise from peritubular cells and subsequently leave the lamina propria to gain access to the interstitial tissue (Kerr et al, 1985). During pubertal maturation, new Leydig cells apparently differentiate from peritubular interstitial cells (Christensen, 1975; de Kretser, 1967; Fawcett,

1973; van Straaten and Wensing, 1978), and this region is also the site of development of numerous Leydig cells 4 weeks after EDS treatment (Kerr et al, 1985).

Mast cells are a free cell-type usually found in connective tissues, and their presence has been confirmed in interstitial testicular and epididymal tissues, as well as the testis mediastinum and tunica albuginea (Hermo and Lalli, 1978; Nistal et al, 1980; Nykanem, 1980; Maseki et al, 1981). Nistal et al (1984) have shown that mast cells of the testis and epididymis are similar to those found in other connective tissues. The number of mast cells increases in infancy, decreases in childhood, and increases again at puberty. During adulthood, mast cell numbers progressively decline in all testicular and epididymal connective tissues, although Maseki et al (1981) reported an increase in numbers (Mastocytosis) in patients with idiopathic male infertility. The increase in mast cell numbers in the normal individual correlates with development of testicular connective tisses, and Mast cell degranulation and heparin release is concommitant with collagen synthesis (see Nistal et al, 1984). At the beginning of puberty, proliferation of active fibroblasts occurs, coinciding with increased gonadotrophin levels and Leydig cell differentiation. The increase of Mast cell numbers during this time may be of importance for developing Leydig cells given their inevitable association in the interstitium.

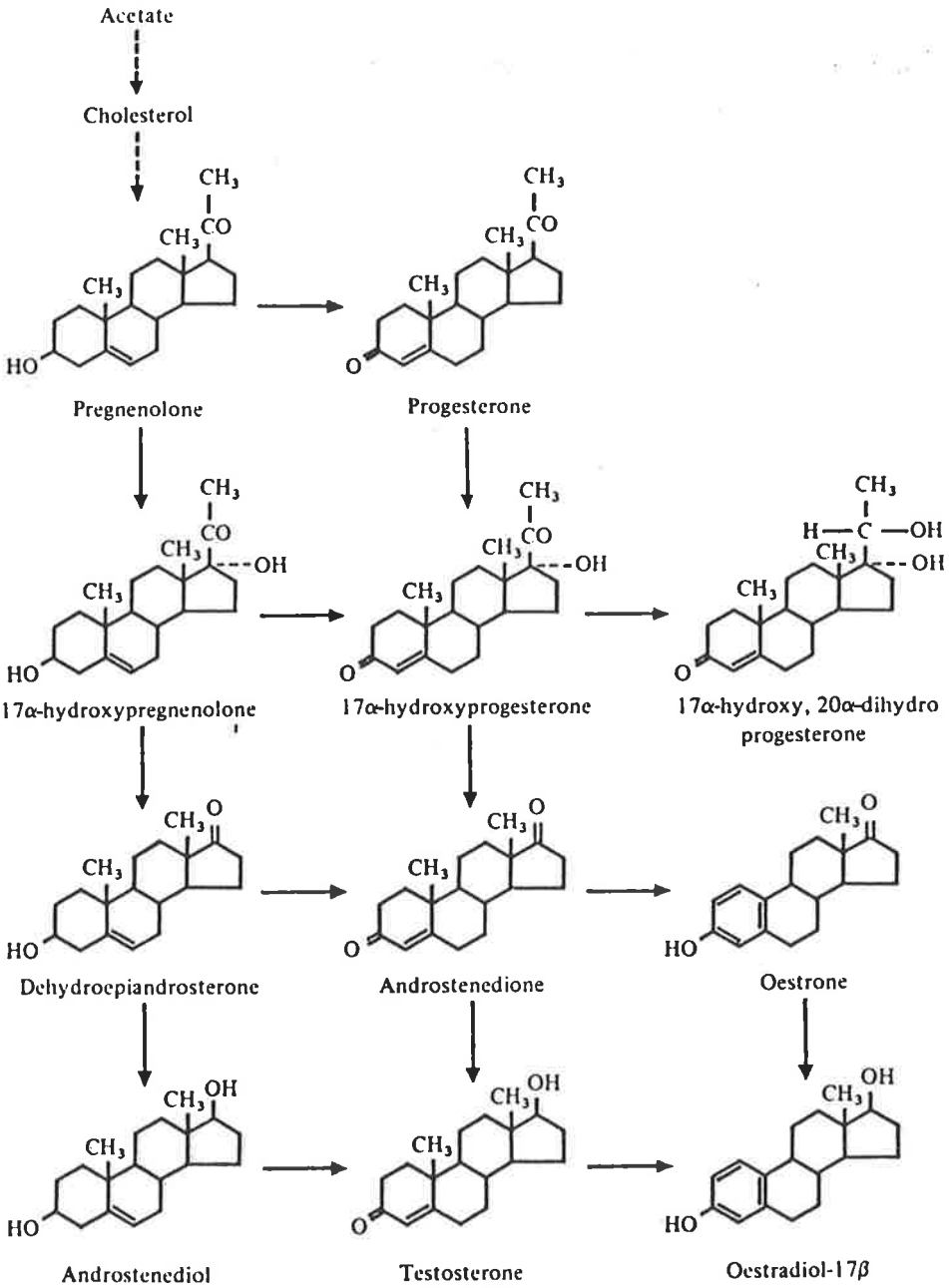
1.2.2.4. Hormones produced by the Leydig cells

That the Leydig cell is the predominant source of androgen in the mammalian testis has already been addressed in this review. However a

variety of steroids are synthesised in the testis from either cholesterol, formed elsewhere in the body and transported to the testis in blood; or acetate, derived from the blood or formed as acetyl-coenzyme A during metabolism of glucose (Setchell, 1978). Testosterone is quantitatively the most important steroid secreted by the testis, and produced by the Leydig cells. That a number of other steroids can be measured in the testis is not surprising when one examines the pathways that allow cholesterol to be transformed to testosterone (see Figure 1.4.). Setchell (1978) has reviewed the endocrinology of the testis, and androstenedione, androstenediol, dehydroepiandrosterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, pregnenolone, progesterone, 5α-dihydrotestosterone, androstanediols, and a variety of 16-unsaturated C_{19} steroids have been found in the venous blood of the testes of a number of species. There is great variation amongst mammals in terms of which steroids are secreted by the testis, and in what concentrations. However it is fair to say that most androgens appear to be produced in the testiscular interstitium, primarily by the Leydig cells.

Prepubertal and fetal Leydig cells appear to produce different products compared to the adult Leydig cell. Hsueh et al (1981) have reported isolated cells of the prepubertal rat testis to secrete androsterone and 5α-androstene-3α,17β-,diol whereas testosterone was the major product in adult cells (Hsueh, 1980). Likewise, the Leydig cell in the sexually mature animal is responsible for testicular oestrogen production whereas the Sertoli cell performs this function in the immature animal (Payne and Valladares, 1980). This has obvious implications for studies on isolated cells since age differences must

Figure 1.4. Outline of the steroid biosynthetic pathways within the testis (from de Kretser, 1984).


Acetate
Cholesterol
Pregnenolone
Progesterone
17α-hydroxypregnenolone
17α-hydroxyprogesterone
17α-hydroxy, 20α-dihydro progesterone
Dehydroepiandrosterone
Androstenedione
Oestrone
Androstenediol
Testosterone
Oestradiol-17β

be carefully considered before any conclusions are drawn about cell function.

Apart from the androgenic products, it is now evident that the Leydig cell synthesizes other bioactive substances that are mainly involved in the paracrine and autocrine regulation of tesicular function. Early studies by Niemi and Kormano (1965) demonstrated a possible role for Oxytocin in mediating contractility of the seminiferous tubules, and since then Oxytocin and Vassopressin have been found in small amounts in testicular material from rats, men and bulls (Wathes, 1984) together with Neurophysin suggesting local biosynthesis. Recently Guldenaar and Pickering (1985) have demonstrated immunocytochemical localisation of oxytocin in rat Leydig cells, although they found no evidence of vasopressin or neurophysin.

Synthesis of prostaglandins has also been demonstrated in the testis, although their precise effects and role in testicular tissue remain to be clearly defined. Haour et al (1979) found an increased secretion of prostaglandin $F_{2\alpha}$ and prostaglandin E_2 by rat Leydig cells, associated with the down-regulation of LH-hCG receptors after hCG administration. Carpenter et al (1978) investigated prostaglandin production by rat testis tissue, and found $PGF_{2\alpha}$ was primarily synthesised by tubules, while interstitial cells (predominantly Leydig cells) had a greater net synthesis of PGE than $PGF_{2\alpha}$. A number of reports indicate that prostaglandins can directly inhibit LH-induced steroidogenesis in dispersed rat Leydig cells (see Sairam, 1979) which suggests that prostaglandins may have an autocrine/paracrine control function over steroidogenesis. Ellis et al (1975) have proposed an integrated mechanism of synthesis of prostaglandins and testosterone

(see Figure 1.5.), and suggest that a number of alterations seen in the testis after heat, cryptorchidism, or radiation may relate to prostaglandin mediated effects involving cAMP.

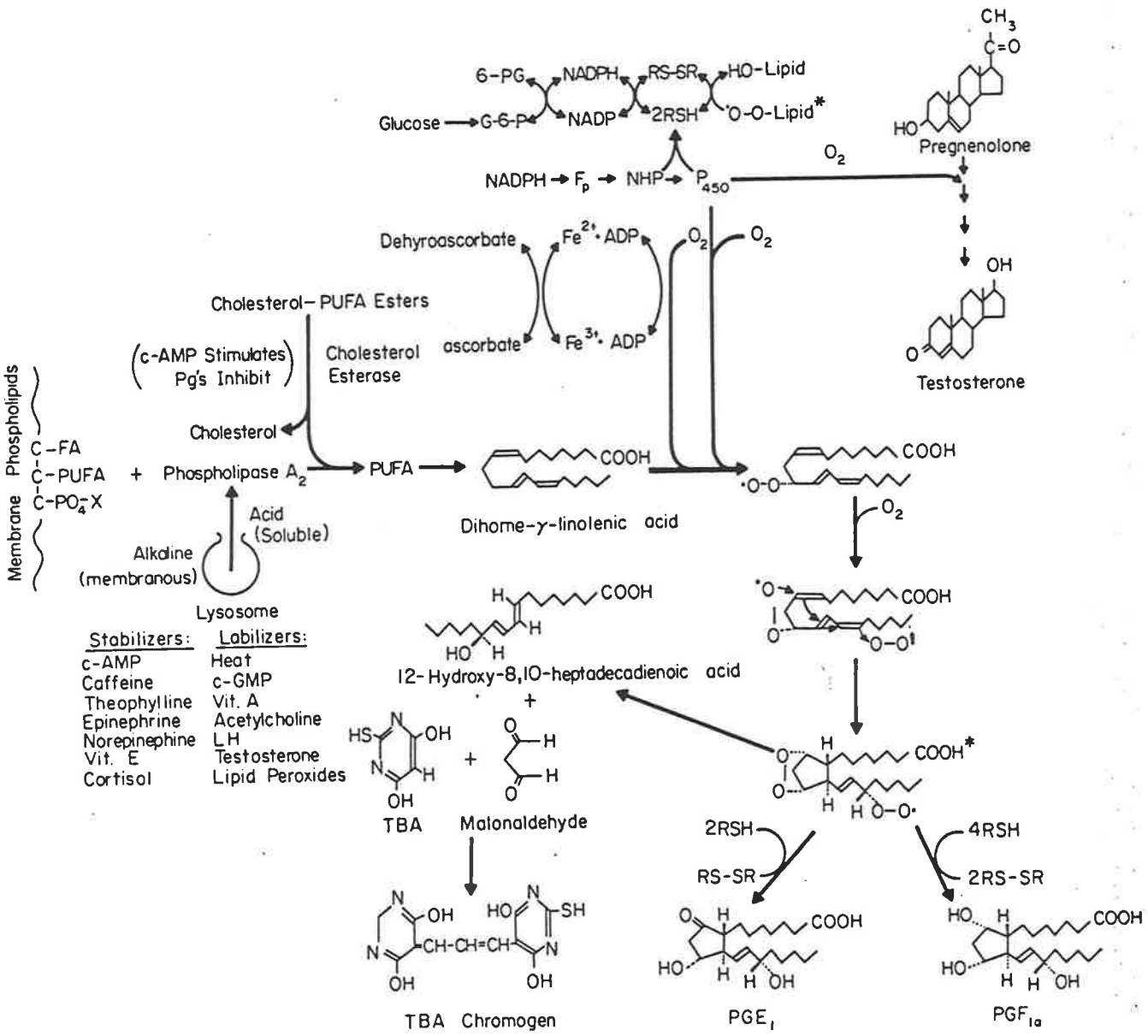
β-endorphin production in rat Leydig cells has been demonstrated (Shaha et al, 1984) and there is some evidence that it may facilitate testosterone secretion either directly as an autocrine effect, or indirectly by way of Sertoli cell-produced or myoid cell-produced intermediates (Bardin et al, 1984). A number of other POMC-derived peptides have also been located in Leydig cells (Margioris et al, 1983) and opiate receptors found in the Sertoli cells by Fabbri et al (1985) suggest that these cells are the targets for testicular opiates. The exact roles for these products remain to be elucidated. However, ACTH and MSH's have been shown to stimulate growth and cAMP accumulation in Sertoli cells and β-Endorphin, possibly with another testicular opiate, inhibits Setoli cell proliferation and ABP secretion (see Bardin et al, 1984).

Parmentier et al (1983) found renin-like immunoreactivity in the testis, and Pandey et al (1984) have found similar activity in purified Leydig cell preparations.

1.2.3. Sertoli cells

In 1865, Enrico Sertoli described columnar cells with cytoplasmic processes extending from the basement membrane to the lumen of the seminiferous tubules, and enveloping the neighbouring germ cells to provide physical support and "nursing" function. Von Ebner (1871, 1888) recognised that the cells of the seminiferous tubules were always found in specific "stages" or associations, and that the

Figure 1.5. Proposed scheme showing the apparent routes for the synthesis of malonaldehyde, Prostaglandin E_1 and Prostaglandin $F_{1\alpha}$, and testosterone synthesis from pregnenolone. Related mechanisms in both pathways involve peroxidation of lipids via either NADPH or ascorbic acid (ADP). See Ellis et al (1975) for further details.


6-PG
NADPH
RS-SR
HO-Lipid
Glucose
G-6-P
NADP
2RSH
O-O-Lipid*
NADPH → F_p → NHP → P_{450}
O_2
Pregnenolone
Dehyroascorbate
Fe^{2+} · ADP
ascorbate
Fe^{3+} · ADP
O_2
O_2
Testosterone
Cholesterol– PUFA Esters
(c-AMP Stimulates
Pg's Inhibit)
Cholesterol
Esterase
Cholesterol
Membrane Phospholipids
C–FA
C–PUFA
C–PO_4–X
+ Phospholipase A_2
PUFA
COOH
Dihome-γ-linolenic acid
Acid
(Soluble)
Alkaline
(membranous)
Lysosome
Stabilizers:
c-AMP
Caffeine
Theophylline
Epinephrine
Norepinephine
Vit. E
Cortisol
Labilizers:
Heat
c-GMP
Vit. A
Acetylcholine
LH
Testosterone
Lipid Peroxides
12-Hydroxy-8,10-heptadecadienoic acid
+
TBA
Malonaldehyde
TBA Chromogen
2RSH
RS-SR
4RSH
2RS-SR
PGE_1
PGF_{1a}

developmental stages of a spermatogenic cycle are arranged spatially in the form of a wave. However he incorrectly concluded that the germ cells developed from the cytoplasm of the Sertoli cells, and that the spermatids degenerated to provide the liquid part of the semen (see Setchell, 1984). The true origin of the spermatozoa and the concept of a symbiotic relationship between Sertoli cells and the developing germ cells was deduced by Merkel (1871), Sertoli (1878) and Renson (1882). While germ cells continually divide, differentiate and are released into the tubular lumen to eventually pass out of the testis, the Sertoli cells are a stable population of cells, which do not divide in the adult animal (Steinberger and Steinberger, 1971; Orth, 1982, 1984). The importance of Sertoli cells in regulating and maintaining the process of spermatogenesis has been emphasised by numerous investigators, although their precise functions and significance for the developing germ cells are still not fully understood.

1.2.3.1. Anatomy

The Sertoli cell rests upon the limiting basement membrane of the semeniferous tubule. The basal portion of Sertoli cells are recognised in tubular cross section as polygonal areas, easily distinguished by their shape and nuclear and cytoplasmic characteristics (Burgos et al, 1970).

The nucleus is infolded in all species studied, being elaborately lobulated in some (Fawcett, 1975). It often lacks both the fibrous lamina that reinforces the inner surface of the nuclear envelope in many cell types, and the karyosome and peripheral clumps of heterochromatin found in the nuclei of most other somatic cell types.

Specialised junctional complexes are formed between neighbouring Sertoli cells, with oppossed membranes approaching to within 20 A in some areas (Dym and Fawcett, 1970). These junctional complexes divide the germinal epithelium into "basal" and "adluminal" compartments, and provide a structural basis for the maintenance of a different milieu in each of these compartments which may be crucial during specific stages of cell development.

The cytoplasm of the Sertoli cells contains long mitochondria. While randomly orientated in the basal cytoplasm, they tend to be orientated parallel to the cell axis in the supranuclear columnar portion of the cell (Fawcett, 1975). Again unlike other cell types, the Golgi apparatus appears to consist of multiple separate Golgi elements scattered throughout the basal cytoplasm and occassionally in the supranuclear region.

Numerous membrane-limited dense bodies are found in the cytoplasm, and include primary lysosomes, autophagic and heterophagic vacuoles, and deposits of lipochrome pigment (Fawcett, 1975). In addition to normal housekeeping lysosomal activities in a cell, the Sertoli cells digest those germ cells that normally degenerate during spermatogenesis, along with lobules of residual spermatid cytoplasm left behind in the release of spermatozoa (Black, 1971; Clegg and McMillan, 1965b; Lacy, 1962).

Rough and smooth endoplasmic reticulum is found in the Sertoli cell cytoplasm, with rough or granular reticulum predominantly in the basal region. This granular form is usually tubular, and while the population of attatched ribosomes is large in the monkey, it is relatively sparse in other species (Dym, 1973; Fawcett, 1975). The smooth endoplasmic reticulum is generally more abundant than the

rough, but again Fawcett (1975) describes significant species differences, with ruminants possessing more extensive quantities than the laboratory rat. The ram Sertoli cell contains aggregates of smooth reticulum in the basal cytoplasm often closely associated with lipid. Localisation also occurs in cytoplasm immediately surrounding developing acrosomes of associated spermatids. Crystalline inclusions have also been described in the Sertoli cell, although they are seldom found in laboratory and domestic animals.

Lipid content is highly variable between species, in both total amount and size of droplets. It is generally localised near the base of cells and appears to vary with the stage of the spermatogenic cycle in some species (Lacy, 1967). After heat, X-irradiation or oestogen treatment which all cause germ cell degeneration, the amount of lipid increases, and Lacy (1967) has speculated that phagocytosis by Sertoli cells of germ cell residual bodies may contribute to this lipid pool, which in turn might be utilized by Sertoli cells for steroid synthesis. The abundance of smooth endoplasmic reticulum suggests that Sertoli cells may have the capacity for steroid biosynthesis (Steinberger and Steinberger, 1977).

At certain stages of the spermatogenic cycle, microtubules are abundant in the Sertoli cell cytoplasm. While considered to be mainly cytoskeletal elements providing support for the columnar portions of the cell, they may also be actively involved in movements of cytoplasm involved in displacing late spermatids (Russell, 1980).

Unlike the Leydig cell population, which is primarily established in the adult animal at puberty; the mitotic activity of the Sertoli cell is most pronounced in the fetal and early postnatal period (Orth, 1984). In the rat, Sertoli cell proliferation is maximal on day 20

postconception (Orth, 1982) and falls steadily after parturition, ceasing between the twelth and fifteenth day after birth (Clermont and Perey, 1957; Hilscher and Makoski, 1968; Orth, 1982, 1984). Indeed, the number of Sertoli cells per testis appears to remain unchanged after the second postnatal week, despite some evidence of DNA synthesis in nondividing Sertoli cells (Steinberger and Steinberger, 1977).

Investigations on Sertoli cells in vivo, or using whole testis tissue in vitro are extremely difficult because of the cellular heterogeneity of the tissue. While the germ cell population of the tubules can be depleted by various experimental methods already mentioned, they do not eliminate other cell types (peritubular cells, interstitial cells). Thus like those studying the Leydig cell, numerous workers have attempted to isolate and culture Sertoli cells in vitro. These attempts have been of variable success (see Steinberger and Steinberger, 1977), due mainly to the difficulty in separating the Sertoli cells from other associated cell types. That the mitotic process ceases two weeks after birth in these cells has also restricted the establishment of pure lines and the propagation of cells in vitro, although Mather (1980) has established a clonal cell line (TM_4) and a tumour cell line (TR-ST) with Sertoli cell characteristics (Mather et al, 1982). In the majority of studies, Sertoli cells have been obtained from the prepubertal rat testis (Dorrington et al, 1975; Welsh and Wiebe, 1975; Fritz et al, 1976; Lacroix et al, 1977; Wilson and Griswold, 1979; Wright et al, 1981; Kissinger et al, 1982; Le Gac and de Kretser, 1982; Sharpe et al, 1982). A few investigators have also used Sertoli cells from immature pigs (Vazeille and Chevalier, 1979; Perrard et al, 1985), immature

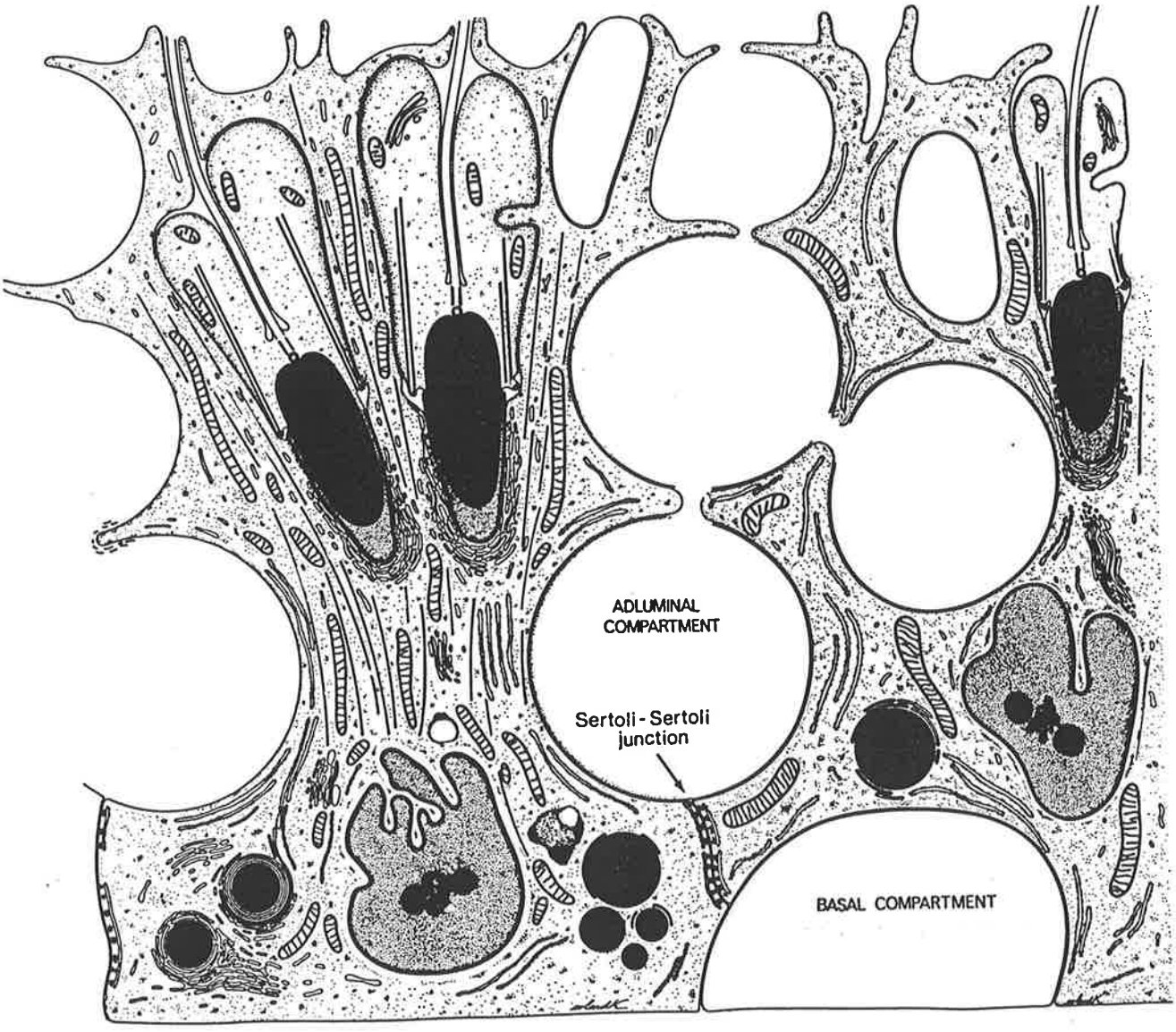
bull (Francis et al, 1981; Vigieri et al, 1985; Hayes, 1986) and immature monkey (Lee et al, 1983). While the culture of Sertoli cells isolated from mature testes has been reported (rat: Steinberger et al, 1975; DeMartino et al, 1977; Cameron and Merkwald, 1981; monkey: Lee et al, 1983) the contamination of such preparations with germ cells remains a problem. While much useful information has been obtained from such studies, it remains to be shown that such cells behave in the same manner as those in the adult animal. Isolation methods of some success utilize a double enzyme digestion of minced testis tissue, with collagenase and trypsin (Welsh and Wiebe, 1975; Dorrington et al, 1975) and produce cell preparations free of Leydig cell and fibroblast contamination. Recently Tung et al (1984) have described an improved isolation procedure using hyaluronidase which yields Sertoli cell preparations of less than 0.3% peritubular cell contamination.

These problems of non-Sertoli cell contaminants are of obvious concern when it comes to interpreting data on cell products obtained from in vitro studies. However a further complication arises from the process of culture itself. Sertoli cells grown on culture plates in defined medium loose their columnar form and adopt a flattened morphology (Suarez-Quian et al, 1984). This implies important cell-cell relationships as I shall discuss shortly. However it has been of concern that cells in such a state may not continue to perform the same functions that columnar cells perform in vivo. Dym and workers have recently cultured Sertoli cells on a reconstituted basement membrane extract in bicameral culture chambers, and in such cultures the columnar form of the cells persists (Suarez-Quian et al, 1984; Hadley et al, 1985; Byers et al, 1986; Djakiew et al, 1986).

1.2.3.2. Relationship to the Blood-testis Barrier

A number of substances of widely varying molecular size introduced into the blood stream rapidly appear in testicular lymph, but not in the fluid collected from the cannulated rete testis (Waites and Setchell, 1969; Setchell, 1970). Such studies defined the existence of a blood-testis barrier, analagous to the blood-brain barrier discovered several decades previously. However unlike the blood-brain barrier which has been found to reside in cell-cell junctions in the walls of cerebral capillaries (Reese and Karnovsky, 1967), the blood-testis barrier is found principally in the walls of the seminiferous tubules. The division of the Sertoli cell epithelium into basal and adluminal compartments has already been addressed in this review. It is the specialised junctions between pairs of Sertoli cells above the spermatogonia, but below the spermatocytes that allows this division, and it is these junctional complexes that are the principle component of the blood-testis barrier (see Setchell, 1978). These sites of restricted permeability have been defined by studies using electron opaque markers (see Fawcett, 1975). They are symmetrical specializations of adjoining Sertoli cells, confined to the basal third of the epithelium (see Figure 1.6.). Just above the spermatogonia, overarching lateral processes of the Sertoli cells meet. In these junctions subsurface cisternae and bundles of filaments develop, and the opposing membranes (normally some 150 - 200 A apart) approach to within 20 A to form the gap junctions. Multiple sites of obliteration of the intercellular cleft are apparent, and it is at these sites that penetrating electron-opaque markers abruptly stop

Figure 1.6. Drawing illustrating the occluding junctions between Sertoli cells. These divide the seminiferous epithelium into basal and adluminal compartments, and form the principle component of the blood-testis barrier (adapted from Fawcett, 1975).


ADLUMINAL
COMPARTMENT
Sertoli - Sertoli
junction
BASAL COMPARTMENT

(Fawcett, 1975). In the immature testis, occluding junctions are absent, and while typical gap junctions are common, they gradually disappear (Gilula et al, 1976). The development of the specialised junctions between Sertoli cells would seem to be under hormonal control since the blood-testis barrier does not appear until the onset of spermatogenesis at puberty (Kormano, 1967a). The unique cell-cell junctions between Sertoli cells are an effective intra-epithelial component of the blood-testis barrier. However, the Sertoli cell has important interactions with other cell types, and the epithelioid cells in the peritubular contractile layer of the seminiferous tubules are now thought to provide an adventitial, albeit incomplete, component to the blood-testis barrier. This is particularly so in rodents, although in larger mammals it may be relatively unimportant, and Fawcett (1975) suggests that in primates, including man, the extensive gaps in the multiple layers of adventitial cells mean that the blood-testis barrier depends exclusively on the Sertoli cell junctions.

The division of the Sertoli cell epithelium by the junctional complexes is of major consequence for fluid partitioning. Substances reaching the base of the epithelium from the blood have more or less direct access to cells in the basal compartment. However the occluding junctions mean that substances must pass through the Sertoli cell cytoplasm to reach germ cells in the adluminal compartment. Likewise the fluid of the tubular lumen must be derived from material passed selectively through, or synthesised by the Sertoli cell (Setchell, 1969). A conspicuous feature of the composition of the fluid of the seminiferous tubule is a high content of potassium (40 mM in the rat; Setchell and Waites, 1975), and Muffly et al (1985) conclude that

Sertoli cells are responsible for this high concentration and that the plasma membrane of these cells contains an active potassium pump.

1.2.3.3. Relationship to other cells

In the intact testis, the mesenchymal cells (peritubular myoid cells and fibroblasts) in the boundary tissue of the seminiferous tubules are adjacent to the epithelial-type Sertoli cells, separated by a basal lamina (Fawcett, 1975) with which only 3% of the Sertoli cell is in contact (Weber et al, 1983). These two cell types have been observed to interact specifically with each other in a complex manner, both in vivo and in vitro (Tung and Fritz, 1986). Peritubular myoid cells of rodents are thought to be derived from fibroblast-like cells in the fetal gonadal interstitium under the influence of adjacent Sertoli cells (Bressler and Ross, 1973). Peritubular cells and Sertoli cells in co-culture interact in a variety of ways resulting in prolonged mutual survival in a medium containing no added protein, under conditions in which neither cell type would otherwise survive in monoculture (Tung and Fritz, 1980). Peritubular cells in co-culture with Sertoli cells sustain the production of Androgen Binding Protein (ABP, a recognised marker for Sertoli cell function; Tung and Fritz, 1980; Hutson and Stocco, 1981), and the addition of peritubular cell conditioned medium to Sertoli cells in culture increases ABP and Transferrin formation (Skinner and Fritz, 1985a). Indeed, peritubular cells are known to secrete a 70 kDa non-mitogenic, paracrine factor termed P-Mod-S which maximally stimulates ABP production in cultured Sertoli cells (Skinner and Fritz, 1986). This indicates that a protein secreted by peritubular cells may be involved in mesenchymal-

epithelial cell interactions between peritubular and Sertoli cells.

These two cell types also co-operate to form components of the extracellular matrix (ECM) and basal lamina of the tubular boundary layer. Cultured peritubular cells secrete collagen and fibronectin, and Sertoli cells in monoculture produce type IV collagen and laminin (Tung et al, 1984; Tung et al, 1985; Skinner et al, 1985). Pollanen et al (1985) have also demonstrated laminin and type IV collagen immunohistochemically in myoid cell layers, and laminin in Sertoli cells of both normal and pathological human testes. The finding by Hermo and Lalli (1978) of mast cells and monocytes as regular components of the walls of seminiferous tubules may relate to collagen synthesis in this region. Proteoglycans constitute important components of the ECM, and are thought to play an essential role in ECM deposition. Skinner and Fritz (1985b) have demonstrated that cultured Sertoli cells synthesize and secrete proteoglycans which contain both chondroitin and heparin glycosaminoglycan chains (GAG chains), while cultured peritubular cells produce proteoglycans of higher molecular mass containing chondroitin GAG but not heparin GAG chains.

As culture and particularly co-culture systems are further developed and improved, the physiological significance of much of this work will become clearer. While the physiological role of peritubular cells, collagen fibrils and other ECM components in the boundary wall in limiting the passage of cells and macromolecules and preventing the penetration of blood vessels is recognised (Dym and Fawcett, 1970), they also play an important role in shielding the immunologically-foreign haploid germ cells from immune surveillance. Hermo and Lalli (1978) reported leukocytes in the the limiting

membrane of the seminiferous tubules in human testicular biopsies, which while found subjacent to Sertoli and germ cells in contact with the basal lamina, were never found in the seminiferous epithelium.

By far the greatest contact between Sertoli cells and other cell types involves the germ cells, and Weber et al (1983) report 84% of the Sertoli cell to be in contact with germ cells. Russell (1980) has reviewed the known interactions between Sertoli cells and germ cells. Sertoli cells show configurational relationships and specialised contacts with developing germ cells. Desmosome-like contacts function as attachment devices to maintain the integrity of the seminiferous epithelium, and at the same time ensure that germ cells are transported in an orderly fashion towards the tubular lumen by virtue of configurational changes of the Sertoli cell. Gap junctions are associated with desmosome-like junctions and are likely avenues for transport of small molecular weight substances from the Sertoli cell to the germ cell population. Elongated spermatids appear to be held within the deep recesses of the Sertoli cell by ectoplasmic specializations, the dissolution of which appears important for sperm release, and seems to allow excess spermatid cytoplasm to be removed from the spermatid head. Sertoli cells phagocytose these complexes just prior to sperm release.

While the Sertoli cell is obviously actively engaged in the spermatogenic process, the inability of investigators to culture germ cells remains highly suggestive that the Sertoli cell provides a milieu essential for germ cell differentiation, and indicates how much more work is to be done in this area. However, co-culture of Sertoli cells with germ cells has been particularly successful (Ziparo et al, 1982; Galdieri et al, 1983, 1984; Tres and Kierzenbaum, 1983) in

studying such interactions. As might be expected in such a close relationship, germ cells also appear capable of signalling to Sertoli cells, and Welsh et al (1985) have reported stimulation of Sertoli cell adenylate cyclase in vitro by germ cells.

1.2.3.4. Hormones produced by Sertoli cells

The possibility that the seminiferous tubules might possess steroidogenic functions was suggested by the occurrence of tumours believed to originate from Sertoli cells, which secreted oestrogens (Huggins and Moulder, 1945). The abundance of smooth endoplasmic reticulum in the Sertoli cells, similar to other steroid producing cells, made this suggestion plausible on a morphological basis (Christensen and Fawcett, 1961). However, there has been some controversy over what hormones are actually produced. While Christensen and Mason (1965) found significant androgen biosynthesis by isolated seminiferous tubules in vitro, it seems likely that these preparations were contaminated by interstitial cells. Cooke et al (1972) incubated interstitial tissue in vitro and found the content of testosterone increased with time, whereas the amount of testosterone in isolated tubules decreased during incubation or was unchanged. However, de Jong et al (1974) found the oestradiol content of separated tubules increased during incubation, while that in interstitial tissue did not change.

Sertoli cells cultured from the testes of prepubertal rats formed appreciable amounts of oestradiol-17β and oestrone when testosterone, 19-hydroxytestosterone, androstenedione or 19-hydroxyandrostenedione were added to the medium. However this activity was greatest in cells

from 5 day old rats, and had fallen to undetectable levels in cells cultured from 30 to 40 day old rats (Armstrong et al, 1975; Dorrington and Armstrong, 1975; Dorrington et al, 1976). While the tubules may have the capacity for converting progesterone and pregnenolone to a number of compounds including both testosterone and androstenedione, and other compounds not formed by the interstitial tissue, they do not appear able to synthesise androgens from cholesterol, which remains a function of the interstitial tissue and primarily the Leydig cells (Setchell, 1978).

Oestrogen production by the Sertoli cell is now thought to be important in regulating Leydig cell activity. A direct effect of oestadiol on testosterone synthesis in the testis has been demonstrated by a number of laboratories, and oestrogen receptors are found in interstitial cells (see Steinberger and Steinberger, 1977). The ability of androgens and oestrogens to differentially influence LH and FSH secretion has also been reported (Steinberger and Chowdhurry, 1974; Steinberger and Chowdhurry, 1977).

However, the hormone produced by Sertoli cells which probably has the most chequered history of all reproductive hormones is inhibin. While the existence of inhibin was postulated over 60 years ago, it has only recently been isolated and characterised. Its name was coined by McCullagh (1932) for a non-steroidal substance, now known to be a glycoprotein, that feeds back on the anterior pituitary to specifically decrease the secretion of FSH (see reviews by Setchell, 1980; Steinberger, 1983; de Jong and Robertson, 1985). By its inhibiting effects on FSH secretion, it can alter spermatogenesis (see de Jong et al, 1978) and while this probably remains the major role for inhibin in the male, it may exert other effects within the testis.

Franchimont et al (1981) found a dose-dependant inhibitory effect on the division of germ cells (see also Demoulin et al, 1981). Nagendranath et al (1982) report that dihydrotestosterone inhibits the release of inhibin by Sertoli cells, and Hurkaldi et al (1984) suggest that testosterone concentrations may regulate inhibin levels.

Inhibin has recently been shown to be composed of an interlinked α- and β- subunit, and Vale et al (1986) and Ling et al (1986) have shown that dimers of the β-subunit can form (which complicates any immunoassays of this hormone where the antisera is directed against the β-subunit), and that these dimers are potent stimulators of FSH synthesis and secretion, which now raises the possibility of dual control of FSH from within the gonads (Tsonis and Sharpe, 1986). Just how this dimer interacts at the level of the pituitary with LHRH remains to be determined.

There has also been considerable interest expressed recently in paracrine substances within the testis (Sharpe, 1986; Tahka, 1986) and I have already touched on some known interactions between Leydig cells and seminiferous tubules. However a number of factors are now being located in the testis that are either unique to this organ, or similar to substances found elsewhere in the body but which possess specific paracrine roles in the testis. Sharpe (1984) has reviewed the known intragonadal hormones, and gonadal LHRH is the hormone with the most extensive bibliography. There is evidence for the Sertoli cell origin of a testicular LHRH-like peptide (Sharpe et al, 1981) and it likely acts as a communicator between Sertoli and Leydig cells to control intratesticular testosterone levels (Sharpe et al, 1982). While testosterone plays a central role in driving spermatogenesis, there is growing evidence for the production and action of peptide growth

factors which may mediate the gonadotrophic and steroidal effects, and Feig et al (1983) have isolated one such factor from Sertoli cells.

Recently Benhamed et al (1985b) have reported FSH regulation of Leydig cell function via at least two types of Sertoli cell generated proteins, and Perrard-Saperi et al (1986) report a direct Leydig cell regulation of Sertoli cell function highlighting yet again the ultimate importance of co-culture and in vivo studies, and the consideration of paracrine control and interactions.

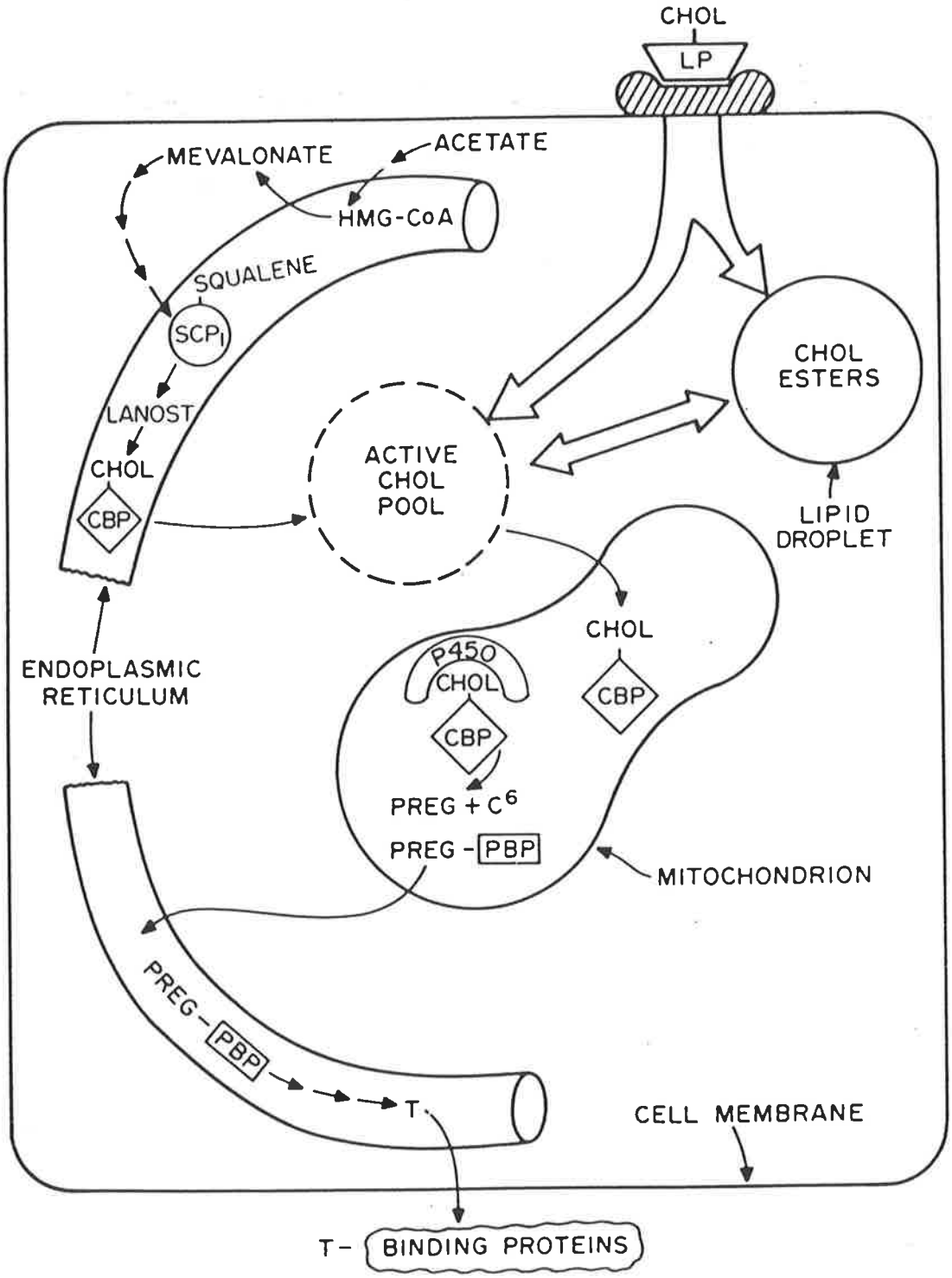
1.2.4. Mechanism of transport of hormones out of and across cells

A number of different cells in the testis produce a variety of substances that act within the testis and elsewhere in the body generally, necessitating the secretion and transport of these products in a manner which allows their action to occur in the right place and at the right time. Such processes must also be carefully regulated. Just as the mechanisms of action of steroid and protein hormones in target cells are different (see Mainwaring, 1977; Catt et al, 1980) so their production and mechanism of transport differ. Of the testicular steroids, testosterone is by far the most studied.

1.2.4.1. Hormone Secretion

Cholesterol is produced by the Leydig cell smooth endoplasmic reticulum, passes to the mitochondria for side-chain cleavage and conversion to pregnenolone, which then returns to the smooth endoplasmic reticulum for conversion to testosterone (see Figure 1.7.). How this then leaves the cell is unknown (see Christensen,

Figure 1.7. The spatial organisation of the testosterone biosynthetic apparatus in a Leydig cell. Cholesterol (CHOL) in the metabolically active pool is derived from de novo synthesis, cholesterol esters in lipid droplets, or in blood plasma. Cholesterol probably is transported from the blood plasma into the Leydig cell by lipoprotein (LP), which may bind to Leydig cell membrane receptors. Cholesterol biosynthesis takes place in the cytoplasm and endoplasmic reticulum. Cholesterol binding proteins (CBP) may exist to transport cholesterol to the mitochondria for conversion to pregnenolone. Pregnenolone binding proteins (PBP) are then thought to return the pregnenolone to the endoplasmic reticulum for conversion to testosterone. How this then leaves the cell is unknown, although binding proteins may be involved (from Ewing and Zirkin, 1983).


CHOL
LP
MEVALONATE
ACETATE
HMG-CoA
SQUALENE
SCP1
LANOST
CHOL
CBP
ACTIVE CHOL POOL
CHOL ESTERS
LIPID DROPLET
ENDOPLASMIC RETICULUM
P450 CHOL
CBP
CHOL
CBP
PREG + C6
PREG - PBP
MITOCHONDRION
PREG - PBP
T
CELL MEMBRANE
T - BINDING PROTEINS

1975). Steroids and their substrates are soluble, and cannot be localised by conventional techniques such as autoradiography. The expected mode of secretion depends on whether the newly synthesised testosterone is released into the cytoplasm outside the tubules of the smooth reticulum; into the hydrophobic portion of the reticulum membrane itself, or into the cavity of the smooth reticulum. Christensen (1975) discusses the implications of each of these possible mechanisms, on the mode of testosterone secretion. Simple diffusion is unlikely to occur, and it is more than likely that a carrier protein is involved, possibly in association with the Golgi bodies. Whatever the mode of secretion, testosterone eventually emerges from the Leydig cell into the extracellular space, possibly bound to a carrier protein (which would increase its solubility).

Protein hormone secretion by testicular cells is unlikely to be any different to the mechanism employed by other protein secreting cells. Studies of the pancreas (Jamieson and Palade, 1967a,b) demonstrated that after protein synthesis on the rough endoplasmic reticulum, the product passes promptly into the cavity of the endoplasmic reticulum and is then transported in solution to the periphery of the Golgi complex. There small membrane vesicles bud off the reticulum and carry the protein into the Golgi region where they are fused with condensing vacuoles. Water withdrawal from the vacuoles concentrates the protein therein. The resulting secretory granule is transported to the surface of the cell where its content is released into extracellular space (Christensen, 1975).

1.2.4.2. Transport

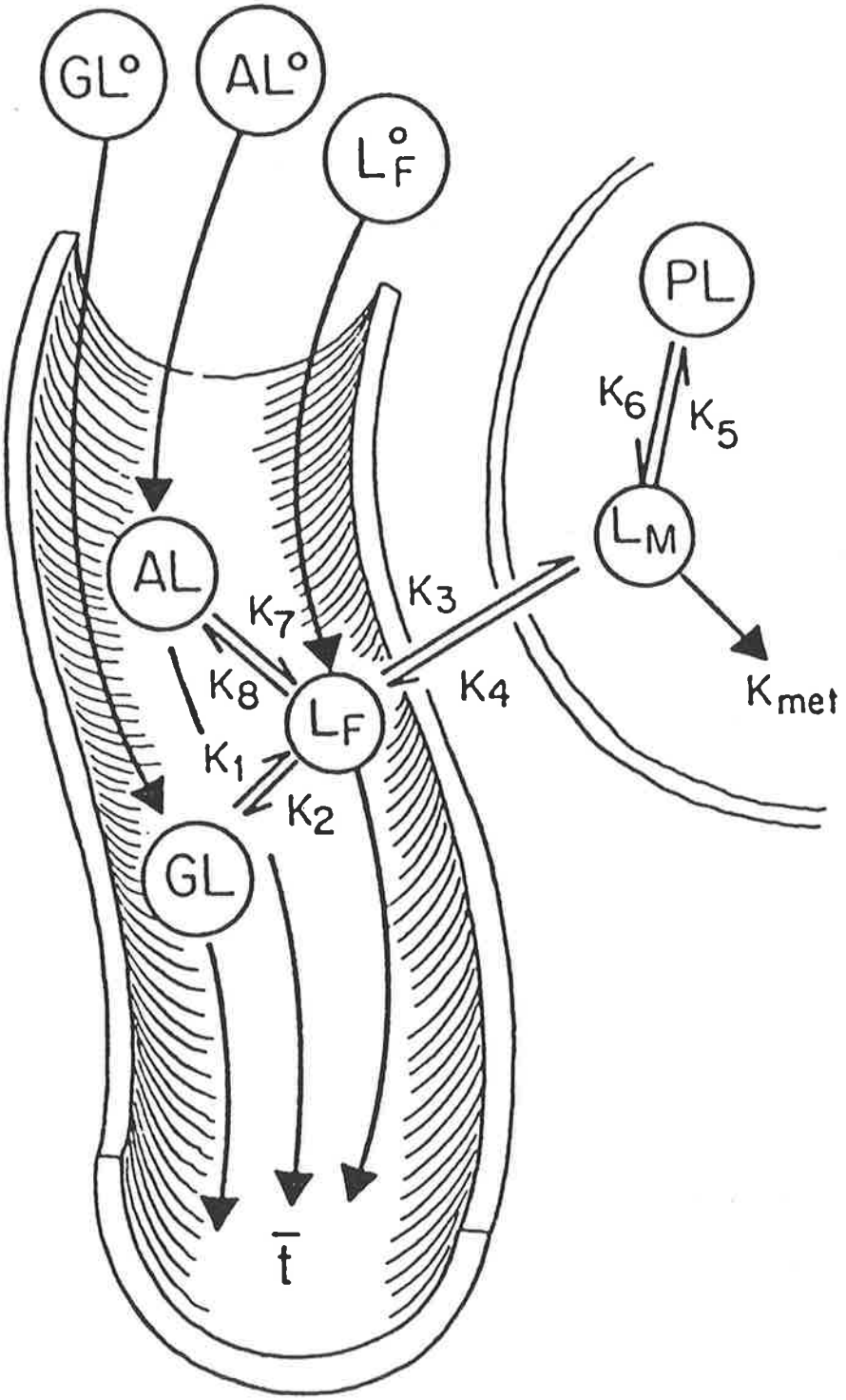
Hormones secreted into the interstitial tissue leave the testis by one of three routes : by entering a capillary, by passing into a lymph vessel, or by traversing the myoid cell layer to enter a seminiferous tubule. As discussed earlier, the great species variation in interstitial anatomy probably affects the relative partitioning of testosterone between lymph and blood. In those species possessing large lymphatic sinusoids (rodents), the lymphatic route is envisaged as the primary source of androgen supplied for the seminiferous tubules, while those animals with more clearly defined lymphatic vessels (sheep, humans) have blood capillaries in much closer contact with the tubules which may directly supply testosterone (Fawcett et al, 1973). While androgens are found in the lymph of all species, and testicular lymph contains much more testosterone than testicular arterial blood (Lindner, 1963, 1967, 1969) blood is the major route of transport for testosterone from the testis to the rest of the body. This is primarily because of the hundred-fold difference in flow rate (more than 1000 ml of blood per hour compared with about 10 ml of lymph per hour; Setchell, 1978). Blood in the deferential vein however contains more testosterone than arterial blood, and blood from arteries in the spermatic cord may contain more testosterone than arterial blood elsewhere in the body because of vein to artery transfer (Free and Jaffe, 1975; Free and Tillson, 1975).

A significant proportion of testosterone in blood is bound to plasma protein, including albumin and corticosteroid-binding globulin (Eik-Nes et al, 1954; Eik-Nes, 1970). A sex hormone-binding globulin (SHBG) distinct from cortisol-binding globulin (CBG) is present in the

plasma of several species including man, but is not found in the rat or boar (Corval and Bardin, 1973). The affinities of physiologically important steroids for CBG or SHBG are two to three orders of magnitude higher than those for albumin. However albumin is important in determining the magnitude of the non-protein bound or "free" fraction of steroid in plasma (Siiteri et al, 1982). It is generally held that only the free fraction is available to target tissues, and that the level of SHBG and CBG regulate the free hormone concentration with SHBG or CBG-bound hormone forming a pool of readily available hormone that will not be degraded by the liver. However, Siiteri et al (1982) suggest that plasma steroid binding protein may play an essential role in the uptake of steroid by target cells, since intracellular CBG has been found in a number of rat tissues. Pardridge and Landaw (1985) have recently modelled testosterone transport in the brain and predict steady-state concentrations of intracellular free hormone to change in parallel more closely to changes in the concentration of bound hormone rather than free hormone, as measured in vitro (see Figure 1.8.). Free (1977) suggests that venous-arterial transfer would also be affected by hormone binding proteins, although the specific interaction of capillary endothelial cells and hormone binding proteins must be considered (Pardridge and Landlaw, 1985).

Lymph is an important route for the secretion of conjugated steroids and some proteins. In pigs and horses, there is appreciably more testosterone, dehydroepiandrosterone and total unconjugated steroids in lymph than in venous blood (Setchell and Cox, 1982; Setchell et al, 1983). Galil et al (1981) found that more than 70% of radioiodinated albumin injected directly into the testis of rams was cleared in testicular lymph. Similar results have been found in rats

Figure 1.8. Steady-state model of testosterone transport through the brain capillary wall and into brain cells. Pools of globulin-bound, albumin-bound and free ligand in the systemic circulation are denoted by GL^o, AL^o, and LF^o, and pools of globulin-bound, albumin-bound, and transportable ligand in the brain capillary are GL, AL and L_f respectively. Pools of free and cytoplasmic-bound testosterone in brain cells are given by L_m and PL respectively. Various rate constants are denoted by K_{1-8}, with the rate constant of hormone metabolism designated K_{met}. This model does not adhere to the restrictions of the free hormone hypothesis and allows for enhanced transport of hormone from the plasma protein-bound pool into the tissue extravascular space. This process is believed to occur via an endothelial inhibition of ligand binding to the plasma protein without the protein crossing the endothelial wall. The model predicts that the steady-state concentration of intracellular free hormone changes in parallel more closely to changes in the concentration of plasma protein-bound hormone as measured in vitro and not the free hormone as measured in vitro (from Pardridge and Landaw, 1985).


GL°
AL°
L°F
PL
K6
K5
AL
K7
K3
LM
K8
LF
K4
Kmet
K1
K2
GL
t̄

(Setchell and Zupp, personal communication). The protein hormone Inhibin is also found in higher concentrations in testicular lymph than in venous blood (see Hudson et al, 1979).

The blood testis barrier restricts the entry of many but not all substances to the seminiferous tubules, and in general lipid solubility appears the main factor determining the rate of entry (Setchell, 1978). Parvinen et al (1970) found exogenous cholesterol penetrated poorly into seminiferous tubules, while progesterone, pregnenolone and testosterone readily passed the blood-testis barrier. While testosterone is found in tubular and rete testis fluid, its concentration is much less than that of the internal spermatic vein, and appears to enter the tubule by facilitated diffusion, involving a saturable carrier (Setchell et al, 1978; Main and Setchell, 1978). Retention and distribution of androgen within the tubules and epididymis seems to involve a specific binding protein in some species. Several mammalian species have been shown to contain a specific Androgen-binding protein (ABP) which likely causes accumulation of androgen in the tubules (Hanson et al, 1975). This protein is produced by the Sertoli cells and enters the epididymis along with other testicular secretions (Danzo et al, 1974). Secretion of ABP is stimulated by FSH and androgen (Fritz et al, 1975; Ritzen et al, 1977). In sheep, proteins generally penetrate slowly through the blood-testis barrier (Setchell et al, 1969) although iodinated ovine LH, FSH and GH entered ram rete testis fluid more rapidly than iodinated albumin or prolactin (Setchell, 1974). In the rat, gonadotrophins penetrated through the walls of the seminiferous tubules but did so very slowly (Setchell et al, 1976).

1.2.5. Composition and function of interstitial fluid

By now it should be apparent that the extracellular or "interstitial" space in the testis is critically important for this organs continued normal endocrine function. The fluid in this region not only bathes the principal cells of the testis but performs the vital role of providing an avenue for communication both between cells within the testis, and between the testis and the rest of the body. Interstitial fluid is always assumed to be formed by filtration of a fluid of low protein concentration at the arterial end of the testicular capillaries and resorption of most of this fluid but not the protein at the venous end of the capillaries. The protein and the rest of the fluid leaves the testis as lymph. While direct evidence for this process has been demonstrated for other tissues (see Yoffey and Courtice, 1970) we can only assume that the system is similar in the testis, where regulation of tissue pressure by contraction of the capsule may be of considerable significance (Setchell, 1986).

For some years now, it has been recognised that all substances entering or leaving the tubules and probably the Leydig cells, must pass through the interstitial fluid. The close association of some Leydig cells with the walls of capillaries may mean that they have a direct association with blood plasma. Indeed, the finding that the concentration of testosterone was higher in the spermatic vein blood than in lymph might suggest a closer association of Leydig cells with blood vessels than with lymphatics. McIntosh (1969) has shown by electron microscopy that Leydig cells may be intimately associated with either, but that there are more blood capillaries than lymph capillaries (see also Yoffey and Courtice, 1970). Likewise, certain

Sertoli cell products may be directly secreted into the tubular lumen. However, the fluid in the interstitial region remains an important avenue for cellular communication, and thus a potentially useful window on what is happening within the testis. Not surprisingly, a number of attempts have been made to monitor the interstitial region.

Practically all in vivo studies have relied on measuring fluids draining the testis (blood, lymph, rete testis fluid), although as may be appreciated by now, the composition of these fluids does not necessarily reflect the composition of fluids within the testis. Lymphatic vessels in the spermatic cord are closely associated with blood vessels, particularly veins, and there is a strong possibility that transfer of material can occur between the various fluids in the cord (Setchell, 1986). This is particularly so for compounds such as testosterone whose production and primary actions both take place in the testis itself. The first real attempt at obtaining interstitial fluid was reported by Pande et al in 1966, using a method that might be termed "Drip Collection". This method involves

> ..." (removing the testes from rats, and making a) small incision at a suitable site through the tunica albuginea avoiding injury to blood vessels and seminiferous tubules. The organs were then placed in a test tube with a small perforation at the bottom and containing a few glass beads. The testes were suspended above the glass beads which prevented blocking of the perforation. The test tube was put inside a conical bottom centrifuge tube into which the fluid from the testes trickled down through the perforation. The operation was carried out at a temperature of 2 ^{o}C under sterile conditions." ...

The collected fluid was centrifuged and the cell free supernatant taken for estimations. While no mention is made in the paper as to how long collection continued, in presenting similar data for the human testis, Pande et al (1967) report collection for 15-17 hours. Since these reports, a number of researchers have used this technique or

variations of it (eg. centrifugation of the whole apparatus for 15-20 min at 54g instead of gravity collection for 15-17 hours) to obtain interstitial fluid for analysis, or for addition to in vitro cultures (see Hagenas et al, 1978; Sharpe, 1979; Sharpe and Cooper, 1983; Sharpe et al, 1983; Turner et al, 1984). However the reported composition of fluid obtained by this method is somewhat unusual for a fluid supposedly formed by blood filtration. While Pande et al (1966, 1967) discuss their results in comparison to blood serum (see Figures 1.9.,1.10.), they make no attempt to explain any of the differences found. In terms of actual ratios the following differences can be highlighted :

	Ratio of IF/Serum		
Substance measured	Rat[1]	Human[2]	Rats + hCG[3]
Protein	2.3/1	1/2	1/1
Lactic dehydrogenase	3.5/1	400/1	
Glucose-6-phosphatase	2/1	3/1	
Glucose-6-phosphate dehydrogenase	1000/1	5000/1	
Acid phosphatase	65/1	112/1	
Ascorbic acid	15/1	30/1	
Glucose	1/7	1/3.5	
Glycogen	5/1	7/1	
Total Lipids	1/3.5	1/3	
Sodium ion	165/152	183/146	140/141
Potassium ion	7/5.6	7.7/5.1	3/1
Chloride ion	125/115	139/115	

1) Pande et al, 1966 2) Pande et al, 1967 3) Sharpe, 1979

A number of the enzymes listed are usually recognised as intracellular enzymes, and together with the high potassium levels are somewhat suggestive of cellular damage and contamination of samples. Apart from the potential problems of the human tissue used by Pande being post-mortem; that the technique of isolating an organ from its blood supply and collecting fluid from it for 17 hours at 2-4 ^{o}C might

Figure 1.9. Biochemical composition of drip-collected rat testis interstitial fluid presented by Pande et al (1966).

TABLE I. Protein, Carbohydrate and Vitamin Constituents of Rat Testis Fluid.

Constituents	Testis fluid	Serum
pH	7.04	7.4
Total protein (g/100 ml)	5.316 (4.31– 6.04)	2.33 (2.2 – 2.5)
Lactic dehydrogenase (units/mg protein/min)	.097 (.09– .10)	.928 (.027–.029)
Glucose-6-phosphatase (Racker units/100 ml)	1.88 (.0 – 2.5)	1.00 (.0 – 3.0)
Glucose-6-phosphate dehydrogenase (units/ml)	987.5 (950–1025)	.0
Acid phosphatase (mgP/100 ml/hr)	65.5 (59.2 –71.8)	.0
Alkaline phosphatase (mgP/100 ml/hr)	63.3 (59.8 –64.5)	64.6 (63.1 –69.3)
Hyaluronidase (units/100 ml)	.0	.0
Carbonic anhydrase (units/ml)	2.0 (2.0 – 2.0)	.0
Glucose (mg/100 ml)	9.0 (8.5 –10.3)	63.7 (56.6 –69.0)
Glycogen "	17.2 (15.0 –19.6)	3.15 (2.5 – 4.0)
Lactic acid "	800.0 (700–900)	1.37 (1.15– 1.7)
*Sialic acid "	15.0	35.0
Ascorbic acid "	10.25 (9.62–10.87)	.69 (.69– .69)

* Data based on a single estimation.

TABLE II. Lipids and Electrolytes of Rat Testis Fluid.

Constituents	Testis fluid	Serum
Total lipids (mg/100 ml)	129.31 (126.6 –131.14)	405.2 (400.21–406.10)
Free sterols "	11.16 (10.24– 13.37)	28.5 (26.2 – 34.6)
Sterol esters "	37.79 (36.14– 38.50)	59.21 (56.37– 61.24)
Phospholipids "	15.65 (12.02– 18.12)	128.2 (126.8 –129.6)
Choline (% of phospholipids)	36.81 (32.25– 36.93)	46.62 (44.31– 49.20)
Serine "	3.64 (3.01– 4.32)	8.22 (7.9 – 8.26)
Ethanolamine "	10.19 (9.22– 10.97)	24.12 (23.93– 26.14)
Sodium (mEq/l)	165.17 (163.27–170.75)	152.17 (146.0 –153.12)
Potassium "	6.9 (5.4 – 7.5)	5.6 (5.2 – 5.9)
Chloride "	125.0 (119.2 –126.3)	115.0 (112.1 –118.2)
Calcium "	2.27 (1.91– 2.49)	5.3 (4.7 – 5.7)
Bicarbonate (mM/l)	23.32 (20.07– 25.69)	13.14 (9.0 – 14.07)
Zinc (μg/100 ml)	20.0 (18.0 – 23.0)	80.0 (75.0 – 85.0)

Figure 1.10. Biochemical composition of drip-collected human testis interstitial fluid presented by Pande et al (1967).

TABLE 1. Protein, carbohydrate and vitamin constituents of human testis fluid.

Constituents	Testis fluid	Serum
pH*	7.0	7.5
Total protein (g/100 ml)	3.71† (3.60-3.77)	7.18 (6.92-7.45)
Lactic dehydrogenase (U/mg protein/min)	0.251 (0.243-0.259)	0.0007 (0.000-0.0007)
Glucose-6-phosphatase (Racker unit/100 ml)	6.3 (3.0-9.0)	2.3 (1.0-3.0)
Glucose-6-phosphate dehydrogenase (U/ml)	5250 (4950-5550)	0.0 (0.0-0.0)
Acid phosphatase (mg P/100 ml/hr)	112.4 (103.6-116.8)	0.0 (0.0-0.0)
Alkaline phosphatase (mg P/100 ml/hr)	19.23 (16.7-20.5)	4.5 (2.7-5.4)
Hyaluronidase (U/100 ml)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Carbonic anhydrase (U/100 ml)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Glucose (mg/100 ml)	21.1 (20.4-22.5)	73.0 (71.0-74.0)
Glycogen (mg/100 ml)	20.2 (19.6-21.0)	3.0 (2.5-3.25)
Lactic acid (mg/100 ml)	600.00 (544-639)	46.8 (42.8-49.6)
Ascorbic acid (mg/100 ml)	30.9 (28.0-34.0)	0.314 (0.146-0.482)

* Determined by BDH indicator paper.
† Mean, with range in parentheses.

TABLE 2. Lipids and electrolytes of human testis fluid

Constituents	Testis fluid	Serum
Total lipids (mg/100 ml)	281.32	656.25
Free sterols (mg/100 ml)	42.11	57.36
Sterol esters (mg/100 ml)	79.23	137.15
Phospholipids (mg/100 ml)	19.93	246.55
Sodium (mEq/l)	183.12	146.33
Potassium (mEq/l)	7.76	5.12
Chloride (mEq/l)	139.65	115.37
Calcium (mEq/l)	3.78	5.71
Bicarbonate (mEq/l)	28.67* (25.44-30.36)	23.44 (22.61-25.93)
Zinc (μg/100 ml)	70 (55-80)	130 (125-133)

* Mean followed by range in parentheses; other data based on a single estimation.

be "unphysiological" was never addressed. The low glucose levels, and the high glycogen and lactate levels might also be indicative of anaerobic metabolism.

In 1970, Tuck et al employed a micropuncture technique to suck up interstitial fluid from between the tubules. This technique required the removal of at least part of the tunica albuginea which may have disturbed fluid formation and removal. The reported potassium level in samples collected was about 50% higher than blood plasma, whereas Setchell (1970) found potassium levels in spermatic cord lymph to be equal to blood plasma. While equilibration of potassium levels in the lymph collected by Setchell may have already occurred with blood in the spermatic cord, the suggestion of cell damage and contamination of samples by Tuck's technique remains possible. Small volumes were also a drawback with the technique.

Interstitial testosterone concentrations have been reported by a number of workers using the Drip collection method, and Comhaire and Vermeulen (1976) were also able to measure testosterone in samples collected by micropuncture (see Table 1.1.). The currently accepted hypothesis that arose from such results, and is well reflected in the table, is that interstitial fluid contains appreciably higher concentrations of testosterone than testicular venous blood (or peripheral blood). However, what is also apparent in this table, and yet never discussed is that the faster the method of recovery of fluid (centrifugation / micropuncture vs gravity) the lower the level of testosterone. This must surely raise further questions about the usefulness or validity of methods of collection.

Of further consideration are the detailed studies of testicular lymph carried out several years ago in the ram. Lindner (1963, 1969),

Table 1.1. The testosterone concentrations in interstitial fluid and venous blood from the testis of the rat, and in peripheral blood plasma. Values are given in ng/ml, with standard errors of the means and the numbers of observations. The values indicated as LHRH-A were from testes which had been injected directly with 1 ng of an agonist of LHRH (see original paper for details).

Author	Technique		Interstitial fluid	Blood plasma	
				Testicular vein	Peripheral
Comhaire and Vermeulen, 1976	Micropuncture		150 ± 27 (17)	-	-
Hagenas et al, 1978	Centrifugation		137 ± 25 (10)	-	1.6 ± 0.7 (10)
Turner et al, 1984, 1985	Centrifugation		73 ± 5 (26)	28 ± 5.3 (10)	1.2 ± 0.1 (8)
Sharpe et al, 1983	Gravity	Control	315 ± 29 (4)	-	2.7 ± 0.3 (4)
	"	"	590 ± 20 (8)	90 ± 20 (8)	-
	"	LHRH-A	800 ± 30 (8)	175 ± 20 (8)	-

Wallace and Lascelles (1964) and Setchell et al (1967) reported testicular lymph in the ram to be similar in composition to blood plasma from the internal spermatic vein. However testosterone levels were approximately 70% of those in blood. Even allowing for lymph/vascular transfer and equilibration, it is extremely difficult to reconcile the reported high concentrations of testosterone in interstitial fluid with lower levels in testicular lymph and venous blood.

It is no longer sufficient to suggest that substances need only be detected in interstitial fluid and that knowledge of specific levels is not all that important. Knowing the actual composition of interstitial fluid is important if we are to understand intercellular communication and the physiological control of testicular function. Much more work is needed in this area, and techniques must be improved if any advances are to be made. It is a region of prime importance in paracrine regulation and communication (Sharpe, 1986). That the majority of infertile men present with normal or near normal gonadotrophin levels and normal or varying degrees of subnormal numbers of sperm (de Kretser and Kerr, 1983) and that we remain unable to provide suitable therapy in most cases reflects our poor understanding of the control of spermatogenesis. Much of this information is probably to be found in the interstitial region, and consequently in interstitial fluid.

1.2.6. Control of endocrine activity in the testis

The endocrine function of the testis has historically been seen

to be controlled by the anterior pituitary, since testicular development and function are so clearly dependent on the gonadotrophic hormones LH and FSH. However it is becoming increasingly evident that the day-to-day functions of the testis are largely under local control (Sharpe, 1983, 1986). I have discussed the role of testicular blood vessels and lymphatics in conveying substances to and from the cells in this organ. However, that these systems can be regulated by the testis is of immense physiological importance, and of prime consideration when discussing the mechanisms of control of endocrine function.

The capillaries of the testis are unusual compared with those of other endocrine tissues in the rat, in that they appear to be unfenestrated (Wolff and Merker, 1966). They also show an extraordinary sensitivity to the toxic effects of cadmium salts (Setchell and Waites, 1970; Aoki and Hoffer, 1978). However their normal permeability would appear to be similar to that of other capillaries in the body as determined by measurements of their permeability-surface area product (PS) for Cr-EDTA, Sodium, Vitamin B_{12} (Bustamante and Setchell, 1981) and Albumin (Setchell et al, 1984). The permeability of testicular blood vessels to albumin can be markedly increased if the animal is injected with human Chorionic Gonadotrophin (hCG) between 8 and 24 hours beforehand (Setchell and Sharpe, 1981; Sowerbutts et al, 1986). The permeability returns to normal between 36 and 48 hours after the hCG. The increase in permeability is accompanied by an increased volume of interstitial fluid within the testis and by increased levels of testosterone in testicular venous blood which peak initially at 2 hours, with further peaks so far recorded at 16 and 72 hours after hCG (Risbridger et al,

1981; Hodgson and de Kretser, 1982, 1984; Hodgson et al, 1983; Sowerbutts et al, 1986). However testicular permeability is normal at the time of the 72 hour testosterone peak suggesting that different mechanisms may be involved in these two effects. The permeability increase does not involve androgens, prostaglandins, histamine or bradykinin, but is mediated by 5-hydroxytryptamine which may arise from the abundant mast cells found in the vicinity of the artery on the surface of the testis (Sowerbutts et al, 1986). HCG is known to bind to LH receptors on Leydig cells and this is the likely stimulus for increased testosterone production by this treatment. However, Bergh et al (1986) and Widmark et al (1986) have produced evidence for the involvement of leukocytes in the permeability response to hCG which supports the above proposition that hCG has more than one mode of action. These effects are of consequence physiologically because LH is reported to have identical effects to hCG on capillary permeability and interstitial fluid volume changes, and is probably normally involved in regulating the tonic control of testicular blood vessel permeabiltiy (see Sharpe, 1983). The testicular LHRH-like substance thought to be secreted by the Sertoli cells appears to not only regulate testosterone secretion, but also to modulate interstitial fluid formation and the results of Sharpe and Fraser (1980) and Sharpe et al (1982) raise the possibility that the delay in action of LH and hCG may in part represent the time taken for substances to induce intratesticular secretion of LHRH.

Just as these changes in blood vessel permeability can dramatically affect the endocrine activity of the testis, so too can the rate of blood flow through these vessels. The vascular system can control the entry or egress of substances into and out of the testis

in two ways. For those substances for which there are concentration gradients across the vascular wall, movement can be regulated by varying the rate of either passive or facilitated diffusion. If this diffusion is not limited, then transport can be regulated by changing blood flow (Setchell, 1986). The finding in the aspermatogenic testis of higher than normal concentrations of testosterone in the testis and in testicular venous blood, but lower than normal levels in the peripheral circulation is probably due to a reduction in testicular blood flow in proportion to the reduction in testis weight (Setchell and Galil, 1983). Testicular blood flow is influenced by a variety of factors including posture, temperature and the common vasoactive substances such as prostaglandins and neurotransmitters (see Setchell, 1970, 1978; Free, 1977). It is unlikely however, that the potential ability of LH to increase testicular blood flow, as discussed earlier, is of any physiological significance since the lag-time for the response is so large, and the resulting change of such small magnitude (Free, 1977; Setchell, 1978). Indeed, the enhanced secretion of testosterone by the ram testis in response to a spontaneous pulse of LH (Laurie and Setchell, 1978) must be due either to a rapid change in permeability of testicular blood vessels or the walls of the Leydig cells, or an equally rapid change in the rate of testosterone synthesis by these cells (see Setchell, 1986). Damber et al (1985) have found the lower secretion of testosterone from the abdominal testis after hCG is mainly due to reduced blood flow and not to any disability of the Leydig cells of abdominal testes to produce testosterone. It remains likely that any effects of LH on testicular blood flow are indirect effects, probably mediated by testicular factors produced in response to LH (such as the LHRH-like factors

described by Sharpe and co-workers), and the suggestion that some product of interstitial cells (Leydig cells, Macrophages) is involved in regulating the neighbouring blood supply (Williams, 1949) requires further investigation.

A blood-testis barrier is probably involved in the control of endocrine activity in the testis. While I have already discussed the major blood-testis barrier in the seminiferous tubules; Kormano (1967b) has demonstrated that at puberty, the penetration of certain dyes into the interstitial fluid is reduced, and some may no longer penetrate at all. This implies some barrier at the level of the vascular endothelium. The barrier does not appear to restrict the passage of most proteins into interstitial fluid (Waites, 1977; Waites and Gladwell, 1982) unlike the blood-brain barrier, and the substances that it is intended to exclude remain to be identified. It is of interest that the establishment of this barrier co-incides with an increase in the activity of alkaline phosphatase, an enzyme considered to be important in transporting substances across capillary walls (Kormano, 1967b). After puberty, the testicular blood vessels are second only to the capillaries of the brain in their level of alkaline phosphatase. While the enzyme is found in the endothelial cells of all blood vessels in the testis, virtually no activity is found in other interstitial cells or the tubules (Setchell, 1986). In contrast, the enzyme gamma-glutamyl transpeptidase which is also present in high concentrations in brain capillaries, is abundant in the endothelial cells of the artery on the surface of the testis and in the spermatic cord and in testicular arterioles, but not in testicular capillaries (Niemi and Setchell, 1986). In the testis, as in other tissues, this enzyme is probably associated with amino acid transport. As emphasised

by Sharpe (1983), the various blood-testis barriers in the testis (vascular endothelium, myoid cells, tight-junctions of the Sertoli cells) will not only selectively prevent certain substances from entering the testis, but may also act to prevent others from leaving the testis. These various barriers allow for the creation of unique microenvironments within the tubules, and in the interstitial space, and are probably essential for local interaction between the various cell types and compartments within the testis.

1.3. THE IMMUNOLOGY OF THE ENDOCRINE TESTIS

1.3.1. Introduction

The fields of reproduction and immunology have historically been classified as separate biological disciplines. A connection between the two was first reported in 1898 when Calzolari observed that the thymus of rabbits castrated before sexual maturity was larger than that of controls. However it took some 70 years before the significance of this observation became apparent and it is only recently that greater emphasis has been placed on interactions between the reproductive and immune systems. It is now recognised that reproductive-immunological interactions are common in mammals. They appear to be hormonally regulated and the hormones involved originate from the thymus, the hypothalamic-pituitary unit and the gonads. A variety of clinical and experimental evidence supports the hypothesis that gonadal steroids regulate immune function (see Grossman, 1985 for a recent review) and the research into the mechanisms involved in maternal acceptance of the immunologically foreign foetus is typical

of work in which the actions of gonadal hormones have been implicated (see Jacoby et al, 1984).

While a significant amount of work has concentrated on the regulation of the thymus and other immmune components by the male gonads, there has also been considerable interest expressed in aspects of immune function within the gonads, and as outlined in the introduction to this review, the immunology of the endocrine testis has proved especially interesting. Along with the brain, anterior chamber of the eye, and the hamster cheek pouch, the testis has long enjoyed a reputation as a peculiarly hospitable site for tissue grafts (Barker and Billingham, 1977). These sites consistently "protect" transplants from the process of immunological rejection usually encountered elsewhere in the body (Billingham and Silvers, 1965; Merrill, 1965) and have been termed Immunologically Privileged Sites.

Before elaborating on the methods of likely protection afforded a graft in such a site, it is necessary to review some basic concepts of transplantation immunology.

1.3.2. The Immune System

The immune system incorporates the lymphatic and vascular systems of the body. The most important cells in the immune system are Lymphocytes. These are basically metabolically quiescent cells which recirculate continuously around the body, passing out of the blood into the lymphatic circulation within the lymph node and other organised lymphoid tissue. They are broadly classified into two main categories

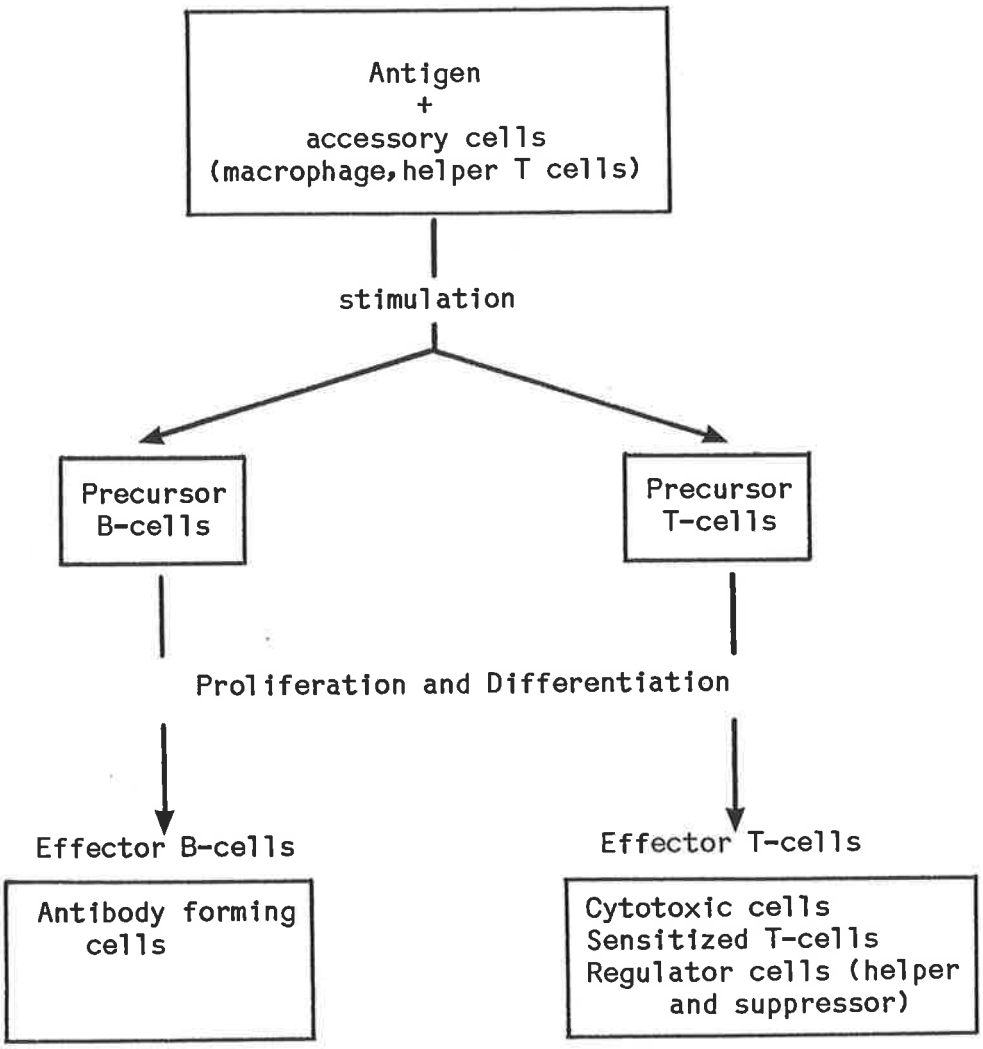
(i) T-lymphocytes : derived from the thymus

(ii) B-lymphocytes : derived from bone marrow in adult mammals

which differ in their developmental background, life span, and functions. They are not distinguishable morphologically, but distinctive surface markers can be detected by immunofluorescence. Lymphoid tissue is a term used to include these cells, together with the primary lymphoid organs where they are produced (thymus and bone marrow in adult mammals), and the secondary lymphoid organs (lymph nodes, part of the spleen, tonsils, Peyers patches in the intestine) where lymphocytes come into contact with antigen and make an immune response (Cunningham, 1978).

Foreign antigens enter the circulating pool and localise in the lymphoid tissue. There they stimulate a proportion of lymphocytes to proliferate and differentiate into "effector" cells (see Figure 1.11.). These effector cells can be antibody producing cells in the case of B-lymphocytes, or a variety of antigen sensitive and regulatory cells in the case of T-lymphocytes. Associated with effector cell production is the generation of Memory cells which join the immune system library as a reference to previously encountered antigens. Antigens stimulating B-lymphocyte proliferation induce antibody forming cells that appear in the stimulated lymphoid tissue within a few days. These cells are usually plasma cells, or basophillic lymphoid mononuclear cells of various sizes, and each produces an antibody of single specificity and of only one immunoglobulin class (Yoffey and Courtice, 1970). This response is called the Humoral Immune Response. The regulatory cells of T-lymphocyte origin are extremely diverse in their function. Helper T-cells interact with B-lymphocytes to stimulate antibody production

Figure 1.11. General pattern of cellular events in an immune response (from Cunningham, 1978).


Antigen
+
accessory cells
(macrophage, helper T cells)
stimulation
Precursor
B-cells
Precursor
T-cells
Proliferation and Differentiation
Effector B-cells
Antibody forming
cells
Effector T-cells
Cytotoxic cells
Sensitized T-cells
Regulator cells (helper
and suppressor)

and Suppressor T-cells depress antibody production. Cytotoxic T-cells seek out foreign cells and interact with macrophages to promote destruction of these foreign cells. These protective functions are referred to as the Cell-Mediated Response. That T-lymphocytes rather than B-lymphocytes or macrophages are primarily responsible for graft rejection has been demonstrated in a number of ways. Nude mice and rats (genetically deficient in T-cells through lack of a thymus) fail to reject skin grafts, and rodents experimentally deprived of T-lymphocytes (thymectomised, irradiated and bone-marrow-reconstituted animals) also fail to reject transplants (Tilney et al, 1981; Kindred, 1979).

When an antigen is introduced into the body for the first time, there is a conditioning of the immune cells or their pre-cursors that extends over a number of weeks, and may result in low levels of circulating antibody. The events occurring in this latent period are described as the Primary response. In the case of a response against a tissue graft which results in rejection, the graft is said to have suffered First-set rejection. If a second exposure to the same antigen or tissue occurs weeks or months afterwards, the memory cells of the immune system generate a rapid production of antibody or cytotoxic cells, with immune response being much quicker than in the primary response. This is called the Secondary response, and in the case of rejected tissue grafts is referred to as Second-set rejection.

Histocompatibility molecules are a class of membrane glycoproteins which are present on the surface of eukaryotic cells and are coded for by a set of genes known as the Major Histocompatibility

Complex (MHC). Histocompatibility molecules belong to two types, one expressed on the surface of all cells, and the other exclusively represented on cells of the immune system. Those expressed on the surface of all cells differ from individual to individual and their genetic diversity provides biological uniqueness for every subject in a normal population. When organs or tissues are transplanted from one individual to another bearing a different MHC coding, they are recognised as antigenically foreign by the host and a rejection response is initiated. Table 1.2. defines the standard terminology used in transplantation immunology. Autografts and Isografts will not differ from the host in MHC coding and will therefore not induce an immune response. Allografts however, while matching the MHC gene loci, will not possess the hosts coding pattern and will likely initiate an immune response. Xenografts usually suffer rejection because their MHC will not only differ in terms of coding pattern , but also gene location. Xenografts will also often suffer from physiological incompatibility which affects survival.

Unmatched graft antigens (allografts, xenografts) reaching the organised lymphatic tissue of the host will trigger a number of processes (for a detailed description see Hayry et al, 1984) of which T-lymphocyte blast formation and division, and the associated production of effector T-cells is of most consequence for graft rejection . A number of different types of T-cells are involved in graft rejection, and they can be differentiated according to membrane expression of particular phenotypes. In mice, one classification system distinguishes lymphocytes by the "Ly" phenotypte. T-lymphocytes expressing the Ly -1,+2 phenotype are directly stimulated by antigens such as tissue grafts and cells bearing tumour antigens or infected

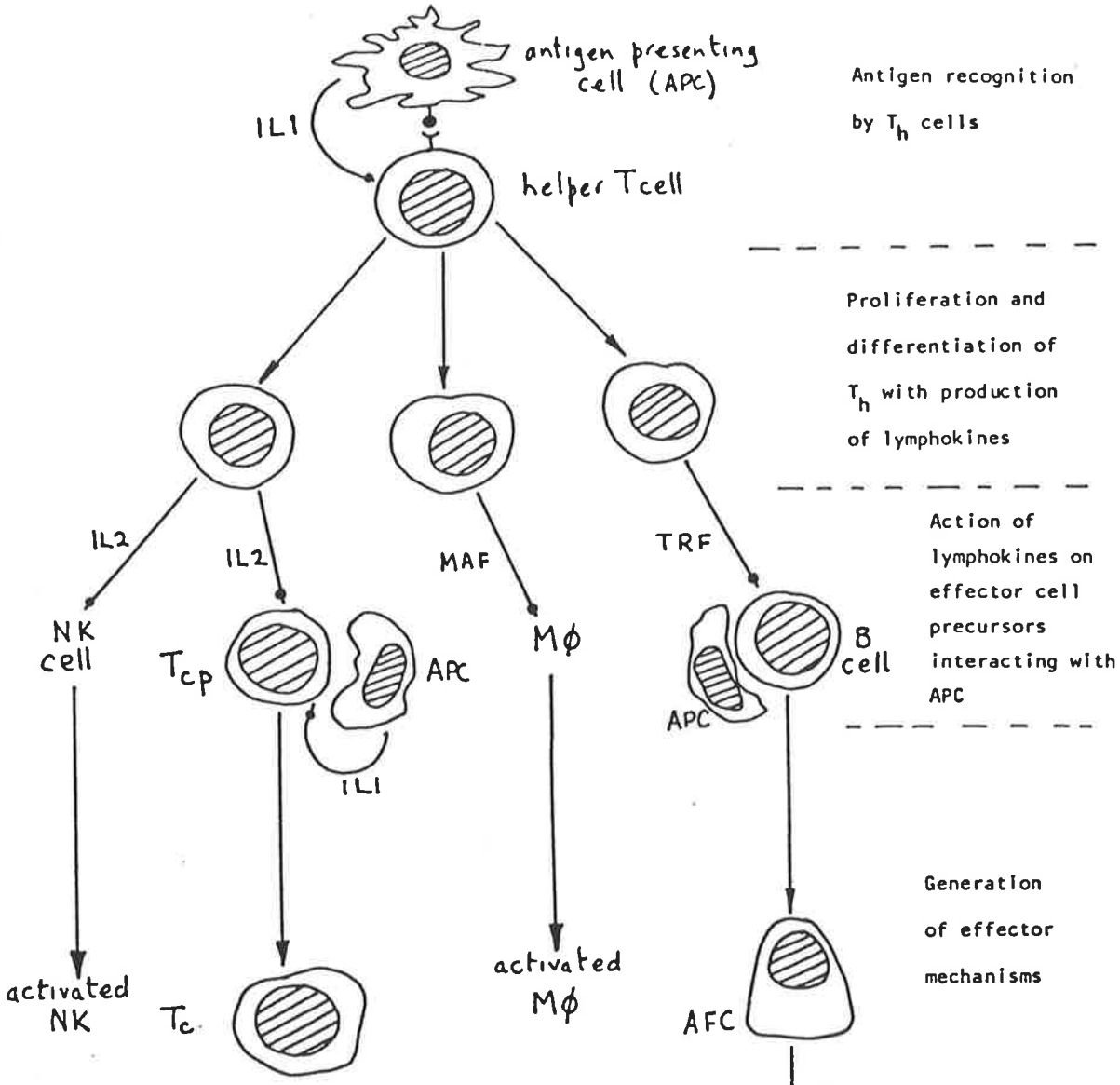
Table 1.2. Terms used in Transplantation Immunology

Prefix	Meaning	Nouns (+ synonyms)	Adjectives (+ synonyms)	Immunogenetic relationship
Auto-	Self	Autograft	Autogeneic (autochthonous) (autologous)	Self to self
Iso-	Same	Isograft	Isogeneic (syngeneic)	Different individuals but genetically identical
Allo-	Other	Allograft (homograft)	Allogeneic	Same species but genetically different
Xeno-	Foreign	Xenograft (heterograft)	Xenogeneic (heterologous)	Different species

with viruses. These T-cells proliferate and differentiate to produce the Cytotoxic or Cytolytic lymphocytes which have the ability to cause lysis of foreign cells. The mechanism of this direct cytotoxicity is poorly understood, but it is specific for the cells that caused immunization, and nearby cellular elements are not affected. Close cell-cell contact is required (Fawcett, 1986). Helper T-cells express a different phenotype, the Ly +1,-2, and enhance the development of Cytolytic T-cells. Transplantation antigens per se, are poor immunogens within a species (Batchelor et al, 1978) and Lafferty (1980) and Bach (1980) suggest that co-presentation is required with a metabolically active lymphoreticular cell. In ordinary parenchymal organ transplants these cells would include blood mononuclear cells and bone-marrow derived "passenger" leukocytes (Steinmuller, 1967; Guttman and Lindqvist, 1969), but not graft parenchymal cells. Support for the co-presenter model comes from a number of authors who have demonstrated that prior culture of tissue makes it possible to transplant skin, thyroid, parathyroid and pancreatic islets of Langerhans across allogeneic or even xenogeneic barriers without immunosuppression. This is suggested to be due to the reduction of immunogenic capacity through the loss of highly immunogenic passenger cells in culture (see Lafferty et al, 1975; Lafferty and Woolnough, 1977; Sollinger et al, 1977; Lacy et al, 1979).

The interaction of an Antigen Presenting Cell (APC) and a Helper T-cell appears central to the whole rejection process, as described by Mason (1983, Figure 1.12.). In this model macrophages / dendritic cells are envisaged as likely antigen presenting cells. Macrophages have the ability to take particulate matter into their cytoplasm and to degrade the ingested substances with hydrolytic enzymes. This

Figure 1.12. The generation of effector mechanisms of allograft rejection (from Mason, 1983).



IL1, interleukin 1 IL2, interleukin 2
MAF, macrophage activating factor
TRF, T cell replacing factor
NK cell, natural killer cell
Tcp, cytotoxic T cell precursor
Tc, cytotoxic T cell MØ, macrophage
APC, antigen presenting cell
AFC, antibody forming cell
ADCC, antibody dependent cell-mediated toxicity
T_h helper T cell

phagocytic function allows them to destroy bacteria, dead cells and debris from injury and they are important in maintaining and repairing tissues, and in defence of the body from invading micro-organisms. They exist as either free macrophages, which are motile and wander through the ground substance of connective tissue, or as fixed macrophages which are sessile, and stretch out along bundles of collagen fibres. It is now widely accepted that both forms are different functional phases in the life history of cells of the same lineage, and all originate from precursors in the bone marrow (Fawcett, 1986). Macrophages actively engaged in engulfing antigen, seem to retain a small amount of antigen bound to their surface which they present in a concentrated form to lymphocytes. However, to be immunogenic such antigen must be presented by macrophages in association with their surface MHC molecules (the Ia molecule in the mouse), and only macrophages with the Ia molecules are capable of co-operating with T-lymphocytes. In lymphoid tissue such as lymph nodes, dendritic cells are likely candidates for antigen presenting cells. They are very similar to macrophages in many ways, but are not phagocytic (Fawcett, 1986).

The Efferent phase of the immune response involves the destruction of the graft by the effector lymphocytes. While sensitized T-cells are an essential first weapon in graft rejection, an exceedingly complex and sizable proliferative response takes place in the graft itself which is associated with that occurring in the lymphoreticular system of the recipient. In the graft, the proliferating cells are mostly lymphoid blast cells or lymphocytes, although some macrophages would also appear to proliferate in situ,

and the overall inflammatory response is now recognised as an important part of allograft rejection. The association of antigen and T-lymphocytes with an antigen-presenting macrophage/dendritic cell causes the APC to release the lymphokine Interleukin-1 (IL-1). This stimulates the Helper T-cell to proliferate and differentiate and appears to induce secretion of a variety of other lymphokines. Interleukin-2 (IL-2) causes proliferation of cytotoxic effector cells, and activated natural killer cells (Nemlander et al, 1983). According to Carpentier (1983), IL-2 is now seen as a major component triggering the immune response in allografts. Indeed, many of the immunosuppressive drugs used in modern organ transplantation programmes to prevent allogeneic graft rejection operate to prevent IL-2 release (see Wang et al, 1982). Macrophage Activating Factor probably stimulates both the macrophage component of the cellular infiltrate, and secretion of plasminogen activator - a neutral protease known to be associated with macrophage effector function (Cohn, 1978; Nathan et al, 1980). Helper T-cells are also involved in the B-lymphocyte generated Antibody Dependant Cellular Cytotoxixity (ADCC) associated with the inflammatory response (see Figure 1.12., and Hayry et al, 1984). The inflammatory lymphocytes, monocytes and macrophages display large numbers of idiotypic receptors to donor MHC antigens, and have a high number of IgG receptors (Roberts and Hayry, 1976; Hayry et al, 1984). Associated with this generation of inflammation is the accumulation of large numbers of thrombocytes in the graft which may contribute in part, to the vascular changes observed in an organ graft vascular tree (Hanto et al, 1982).

It is of interest that an inflammatory episode is often also seen in autografts, although it only superficially resembles that seen in

an allograft. The response is always much smaller, and distinctly lacks blast cells (Hayry et al,1984).

Finally, the concept of transplantation Tolerance requires mention. Immunotolerance is a state of immunologic inactivity that is specific in regard to antigens or cells which, in normal animals would induce an immune response. Cultured allografts that have been depleted of passenger leukocytes, carry recognisable antigen and are promptly rejected if recipient animals are challenged with donor leukocytes at the time of transplantation. However, animals carrying allografts for a prolonged period (>= 100 days) become progressively more resistant to challenge with donor cells (Zitron et al, 1981) and Donohoe et al (1983) have demonstrated that thyroid graft adaption to the host results from the development of tolerance in the recipient. As discussed by Lafferty et al (1983), the induction of tolerance for tissue grafts is likely to develop as a result of the slow leakage of free antigen into the immune system of the recipient. Several mechanisms have been proposed that account for induction and maintenance of tolerance by such a method, resulting in either prevention of T-helper cell activation, or the predominant activation of T-suppressor cells (see Gotze and Mota, 1981). It appears that tolerance is the result of an actively maintained balance between help and suppression and requires the continuous presence of low levels of antigen and antibodies.

Transplantation immunology is a rapidly advancing field in which controversey is widespread, and changes in hypotheses of specific mechanisms of action occur frequently. The above review is an

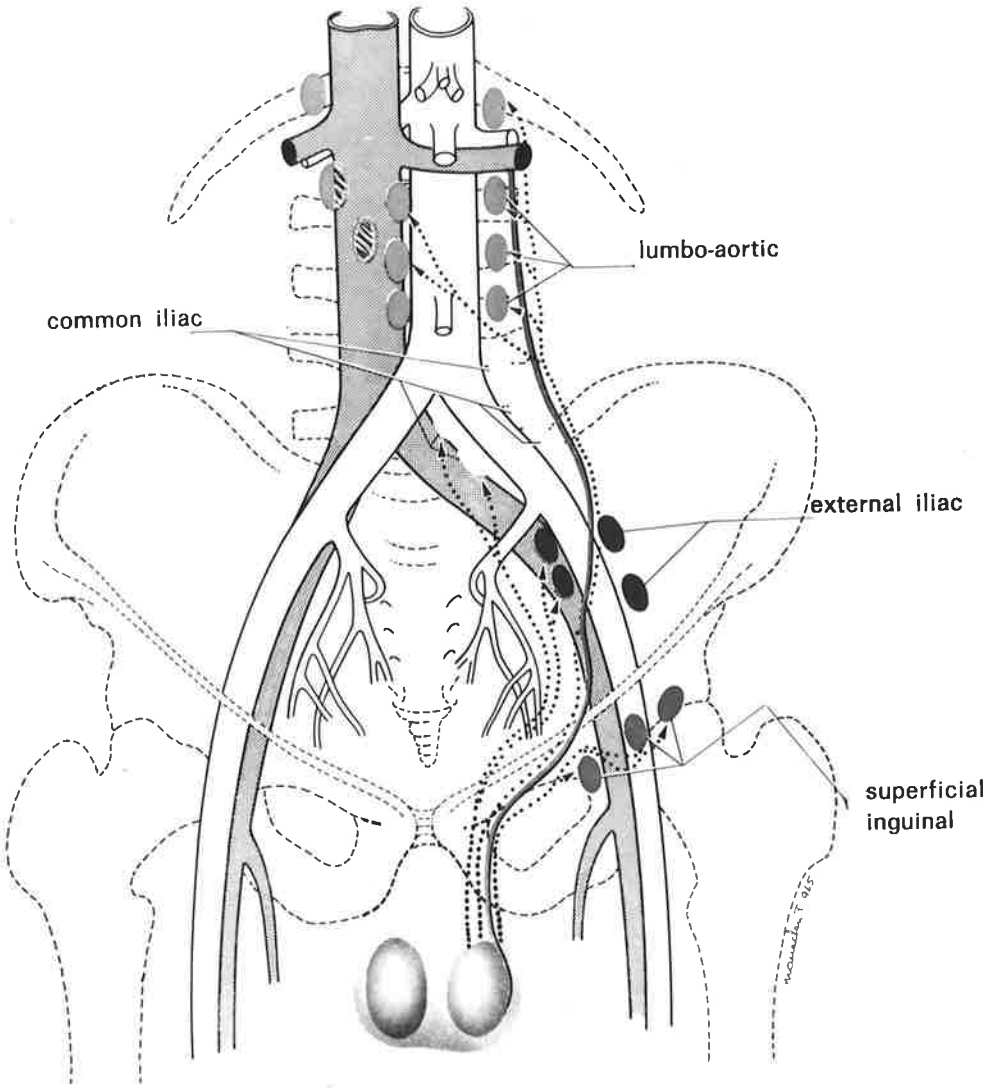
extremely simplistic representation of the mechanisms involved in allograft rejection. However it provides a sufficient foundation on which to proceed with a discussion of Immunologically Privileged Sites.

1.3.3. Immunologically Privileged Sites

The immunologically privileged status of the brain, anterior chamber of the eye, and the hamster cheek pouch has primarily been attributed to their lack of lymphatic drainage (Head et al, 1983a). Without lymphatic drainage there is an effective blockade in the afferent immune response, and this was clarified by Barker and Billingham (1968) when they demonstrated that if skin islands are raised on vascular pedicles with their lymphatic drainage severed, they then become effective privileged sites.

The testis however, becomes a uniquely privileged site in this context, because it is known to possess very effective lymphatic drainage (demonstrated as early as 1830 by Panizza), involving primarily the iliac and renal lymph nodes (Tilney, 1971; McCullough, 1975; Whitmore and Gittes, 1976; Head et al, 1983b). The drainage of the human testis is shown in Figure 1.13. Furthermore, Fawcett et al (1969) and Fawcett et al (1973) have demonstrated that the testicular interstitial connective tissue is laced with a complex fine lymphatic network, as was discussed earlier in this review. Thus, with such a lymphatic connection, other factors must contribute to the immunological privilege afforded the testis, and numerous investigators have attempted to define the mechanism(s) involved. Obviously, if some specific process can be

Figure 1.13. Diagramatic representation of the lymph nodes which collect the lymph of the human testicle and scrotum (from Donini and Battezati, 1972).


lumbo-aortic
common iliac
external iliac
superficial
inguinal

identified in the testis that provides local immunosuppression, without the need to administer immunosuppressive drugs which suppress all immune functions, the consequences for organ transplantation are immense.

1.3.4. The Testis as an Immunologically Privileged Site

The use of the testis in transplantation studies has a long history and in 1771 John Hunter described the transfer of "the testes of a cock into the belly of a hen" (see Forbes, 1947). However studies on the transfer of other tissues into the testis appear to have originated early this century. In 1935, Gardner and Hill published work on the persistence of pituitary grafts in the testis of the mouse, and reported many investigators of the time to have found the testis to be a favourable site for ovarian transplants. From these early reports unfolds a fascinating tale of investigation into the immune privileged status of the testis, the history of which was reviewed by Barker and Billingham in 1977. In 1949, Greene successfully used the testicle in mice, rats and hamsters as a transplant site for Brown-Pearce tumors of rabbit origin. However, he observed that only 50-100% of recipients grew tumors compared with 100% when the same tumor was transplanted to the anterior chamber of the eye. After periods varying from several days to more than a month, the intratesticular tumor grafts regressed and thereafter resistance to reinnoculation of this tumor was noted. Lymphatic extension and metastasis did not occur. Aron et al (1957) also reported successful allo-transplantation of pituitary, thyroid, and kidney tissue to the guinea pig testis, and Medawar and Russell (1958) used adrenal

iso-grafts in mice testes. More recently, Dib-Kuri et al (1975) found that parathyroid allografts sustained normal serum calcium levels in outbred parathyroidectomised rats for longer than 3 months, if placed in the testis. Intramuscular implants in such animals however failed to achieve even transient normocalcaemia, presumably because of early rejection.

The role of histocompatibility was addressed by Naji and Barker (1976) when they studied the fate of parathyroid allografts in parathyroidectomised inbred rats. Survival of intratesticular grafts transplanted across histocompatibility barriers exceeded by many weeks that observed for allogeneic parathyroids in subdermal pockets (12-16 days). However, histocompatibility was a significant factor since only 20% of grafts in the testes of incompatibile hosts survived for 150 days, while 62% of grafts in the testes of a more compatible host survived for this time. Human parathyroid xenografts were rejected so rapidly that normocalcaemia was never seen after intratesticular transplantation in rats. Further evidence that the testis is frequently effective, but <u>incompletely</u> privileged for other tissue allografts comes from the work of Whitmore and Gittes (1975) who found that intratesticular skin allografts in rats were not all protected in this environment with 9 out of 29 grafts showing histological evidence of rejection after 26 days. The findings of Ferguson and Scothorne (1977 a,b), Bobzien et al (1983) and Selawry and Whittington (1984) also support the suggestion that the testis provides effective but incomplete immunological protection. As seen in Table 1.3., a number of other reports also find variability in graft survival times even when the genetic relationship of the host and donor is constant (autografts and isografts).

Table 1.3. Summary of literature reports of intra-testicular transplants.

Tissue/Graft Used	Donor/Recipient Relationship	Graft Duration	% Success Rate	Animals Used	Reference
Adrenal	Isograft	Terminated at 14 days	75	Mice	Medawar and Russell (1958)
Parathyroid	Autografts	> 3 months	100	Rat	Dib-Kuri et al (1975)
	Allografts	> 3 months	100	Rat	
	Xenografts	25 days - > 6 months	90	Guinea Pig -> Rat Rabbit -> Rat	
Parathyroid	Allografts	> 118 days	80	Rat	Nagi and Barker (1976)
Parathyroid	Autografts	Terminated at 6 weeks	85	Rat	Whitmore and Gittes (1979)
	Allografts	Terminated at 6 weeks	80	Rat	
Parathyroid	Isografts	> 400 days	100	Rat	Head et al (1983a)
	Allografts	> 25 days	50	Rat	
		> 200 days	25	Rat	
Pancreatic Islets	Xenografts	Ave. 30 days	100	Rat -> Mouse	Bobzien et al (1983)

cont/...

Tissue/Graft Used	Donor/Recipient Relationship	Graft Duration	% Success Rate	Animals Used	Reference
Pancreatic Islets	Isografts	> 90 days	75	Guinea Pig	Ferguson and Scothorne (1977a,b)
	Allografts	up to 82 days	75	Guinea Pig	
Skin	Autografts	56 days	100	Guinea Pig	
	Allografts	14/21/28/47 days	100/80/15/0	Guinea Pig	
Pituitary	Isografts	Terminated at 3 weeks	85	Mice	Hill and Gardner (1936)
Anterior Pituitary	Homografts	Unknown	100	Guinea Pig	Aron et al (1957)
Thyroid	Homografts	(> 1 week)	80	Guinea Pig	
Ovary	Homografts		Not stated	Guinea Pig	
Papillary Tumour (B240)	Homografts	No take	Nil	Rabbit	Greene (1940)
Acinar Type Tumour (T36)					
- Primary tumour	Homografts	No take	Nil	Rabbit	
- Lymphatic extension	Homografts	No take	Nil	Rabbit	
- Splenic metastasis	Homografts	No take	Nil	Rabbit	
Brown Pearce Tumour	Xenografts	Variable. 12 days to more than 2 months	50-100	Rabbit -> Mice	Greene (1949)
			50-100	Rabbit -> Hamster	
			50-100	Rabbit -> Rat	
			Nil	Rabbit -> G. Pig	

cont/...

Tissue/Graft Used	Donor/Recipient Relationship	Graft Duration	% Success Rate	Animals Used	Reference
Wilms Tumour	Autografts	Terminated at 14 days	100	Wistar Rat	Wajsman et al (1980)
	Allografts	Terminated at 21 days	0	Lewis Rat	
	Allografts + Testosterone/ Oestradiol	Terminated at 21 days	60/80 70/50	Lewis Rat Fischer Rat	
	Allografts	Terminated at 21 days	100	Fischer Rat	
Renal Cell Adenocarcinoma	Autografts	Terminated at 14 days	0	Mice	
	Allografts	Terminated at 21 days	0	Mice	
	Allografts + Testosterone/ Oestradiol	Terminated at 21 days	0/0	Mice	
Neuroblastoma	Autografts	Terminated at 14 days	90	Mice	
	Allografts	Terminated at 21 days	40	Mice	

cont/...

Tissue/Graft Used	Donor/Recipient Relationship	Graft Duration	% Success Rate	Animals Used	Reference
Neuroblastoma	Allografts + Testosterone/ Oestradiol	Terminated at 21 days	40/60	Mice	Wajsman et al (1980)
Insulinoma	Allografts - normal - diabetic	 > 50 days 20-30 days	 6 15	Rat	Akimaru et al (1981)
Teratocarcinomas	Isografts	Unknown	Not Specified "Cells continue to grow and give rise to tumours"	Mice	Hogan (1981)
Placenta	Isografts	Terminated at 5 days	100	Rat	Canivenc (1936)
Vagina	Isografts	Terminated at 1 month	100	Rat	Takewaki (1956)
	Isografts	Terminated at 7 weeks	100	Rat	Takewaki (1957)
Vagina + Ovary	Isografts	Terminated at 2 months	100	Rat	Takewaki (1958)

cont/...

Tissue/Graft Used	Donor/Recipient Relationship	Graft Duration	% Success Rate	Animals Used	Reference
Morula	Allografts	Terminated at 15 days	20	Mice	Billington (1965)
Blastocyst	Allografts		62	Mice	
Ectoplacental cones	Allografts		94	Mice	
Blastocyt	Allografts			Mice	Kirby (1963)
	- scrotal testes	> 14 days	90		
	- cryptorchid testes	> 14 days	94		

1.3.5. Theories for the privileged status of the testis

Once the lymphatic network of the testis was demonstrated, a new theory was needed to support the experimental indications of the immune privilege provided by the testis. The most frequently cited explanation for this privilege was initially proposed by Medawar and Russell in 1958. According to this theory, the lymphatic drainage of the testis is deficient because of an unusually long lymphatic pathway between the organ and the nearest draining lymph node at the level of the kidney (see Plate 1). This theory was challenged by Barker and Billingham (1973) when they showed that skin allografts transplanted orthotopically near the tips of adult rat tails were promptly rejected, despite an afferent lymphatic pathway to the nearest draining lymph node of in excess of 19 centimetres.

In 1959, Engeset reported that in 19 of 65 rats studied, the lymphatic vessels from the testis failed to transverse a lymph node, and passed directly to the thoracic duct. This would account in part for the noted inconsistencies reported earlier, since privilege would only be afforded the testis in those animals in which the testicular lymphatic effluent failed to transverse any lymph node. It was certainly a more plausible theory than that of Medawar and Russell (1958). However, Tilney (1971) and McCullough (1975) have also studied the lymphatic drainage of the testis in rats, and both found that afferent lymphatics led uniformly to at least one subdiaphragmatic lymph node. Whitmore and Gittes (1976) have suggested that the logical existence of an afferent immune blockade is just not applicable, and propose that the afferent arm of the lymphatic system is still active in the testis, with the efferent or effector limb being the arm

malfunctioning. This implies that antigenic material can reach the response-initiating lymph node, but for some reason the effector cells are not produced, or do not return to the graft site.

Ferguson and Scothorne (1977 a,b) demonstrated that while first-set intratesticular grafts rarely induce systemic immunity in the host, second-set intratesticular grafts were usually rejected in the typical rapid second-set fashion, succumbing to a pre-existing systemic immunity generated by first-set skin grafts. This suggests that both arms of the immune system can be activated under the right conditions. Reports of high levels of gonadal infiltration by leukaemic T-cells in cases of acute lymphoblastic leukaemia with primary testicular relapse (Reid and Marsden, 1980; Eden et al, 1978; Tiedmann et al, 1982) also lend support to this suggestion since the efferent immune response must be "active" to allow such infiltration.

It seems in effect, that the immunologically primed male host cannot be regarded as possessing any degree of privilege in the testis (in relation to tissue from the donor against which it is primed), where as the unprimed host does have this privilege, and remains "unprimed" after first-set intratesticular transplantation. This implies that factors in the testis, or between the testis and any given draining lymph node somehow suppress the recognition of testicular-derived antigens, and thus prevent initiation of the immune response.

The studies of Tilney (1971), McCullough (1975) and Whitmore and Gittes (1976) report the investigation of lymphatic connections of the testis, and demonstrate that the lymphatics apparently combine to enter a lymph node without connecting to other organs or vasculature. This suggests that very little happens in the afferent lymphatic

vessels leaving the testis which could prevent antigen recognition in the lymph node. That the given nodes also receive lymph from other sites not considered immunologically privileged (eg. scrotum) means that they are probably not specifically involved in conferring the immune privileged status on the testis. Likewise, the testicular route of exposing the host to antigen is not in itself immunosuppressive as is, apparently, the anterior chamber of the eye where the lack of lymphatic drainage results in exposure of the hosts immune system to graft antigens by the vascular route, with subsequent systemic suppression (Kaplan and Streilen, 1978; Whittum et al, 1982; Streilein et al, 1980). Indeed, parental strain lymphocytes injected into the testes of F_1 hybrid males induce graft-versus-host reactions in the draining lymph nodes, and intratesticular innoculations with allogeneic lymphoid cells result in normal systemic antibody responses (Head et al, 1983c).

Recent evidence that significant events may take place at the site of exposure rather than in the regional lymph node in certain grafting situations is of significance in this regard. Pederson and Morris (1970) employed heterotopic sheep kidney allografts with chronic fistulas in the renal lymphatics to prevent draining antigen or cells from reaching the regional lymph nodes. Nevertheless, the lymph soon contained stimulated lymphocytes, and the graft became infiltrated with lymphoid cells and was promptly rejected. Ascher et al (1981) have shown that cytotoxic precursor cells recruited to sponge matrix allografts, undergo proliferation and maturation to cytotoxic effector cells within the graft site itself.

All the evidence suggests that immune privilege in the testis depends on local factors interfering with these processes to influence

the initiation and development of the rejection response. Although beneficial for graft recipients, the situation may be detrimental for immune surveillance and may explain why the testis, like the brain, is a major site of recurrent tumours in male leukaemic patients (see discussion earlier, and Baum et al, 1979). In the earlier part of this review, I discussed the existance of a blood-testis barrier around the seminiferous tubules. This barrier is capable of excluding from the lumen of these tubules many substances normally present in blood and lymph, including antibodies, and at the same time preventing escape of autoantigenic material associated with maturing components of the germinal epithelium (testicular antigens). While numerous studies on auto-immunity in the male have stressed the importance of this barrier (see Tung, 1980 for a detailed review on this topic), it is unlikely that it plays any direct role in mitigating host reactivity to intratesticular allografts (Barker and Billingham, 1977). Since such grafts are invariably placed in the interstitial region of the testis, this is where attention must be focused. Although the testicular capillaries and venules are unfenestrated, they do allow diffusion of immunoglobulin G (Mancini et al, 1965). As already discussed in detail, the interstitial space contains extensive lymphatics and islands of blood vessels surrounded by Leydig cells, macrophages, mast cells and plasma cells. Resident macrophages have been isolated from the perfused testis (Tung et al, 1979), and the Leydig cell-macrophage ratio is about 4:1. Circulating lymphocytes do reach the testicular interstitium, and from there they enter the lymphatics and can be recovered by cannulae placed into afferent lymphatic vessels (Morris, 1968). Thus, humoral antibody and recirculating lymphocytes attendant on a systemic immune response can readily reach the interstitial space

of the testis (Tung, 1980).

Head et al (1983c) reviewed several possibilities for local suppressive agents in the testicular milieu. The most commonly cited candidate is the Leydig cell, which produces steroids in the interstitial tissue. There is substantial evidence that a number of androgens are immunosuppressive (Clemens et al, 1979; Roubinian et al, 1977) albeit in extremely high concentrations, and the reportedly high levels of testosterone produced by Leydig cells in the testis (certainly higher than levels of this hormone seen elsewhere in the body) have led to speculation on their role as local suppressors of the immune response. However, androgen immunosuppression is a controversial subject (see Cohn, 1979), and other factors need consideration. Leydig cells produce other products such as Progesterone and Prostaglandins, both of which can also be demonstrated to be immunosuppressive. Also, reports that Leydig cells will spontaneously adhere to lymphocytes and nonspecifically suppress proliferation of mitogen-stimulated and alloantigen-stimulated lymphocytes in vitro (Born and Werkerle, 1982) may be of consequence. It remains possible therefore, that suppression of the immune response by local hormones is a feasible explanation for local immunological privilege and Head et al (1983c) suggest that this mechanism may also explain both the increased number of infections and the somewhat prolonged graft survival noted by Frenkel (1961) in the adrenal gland.

The germ cell population itself is another cadidate for providing the immune privilege of the testis. Shearer and Hurtenbach (1982) describe the depressed response of human and mouse lymphocytes to mitogens in vitro in the presence of spermatozoa or their precursors.

When mice are injected intravenously with syngeneic sperm, T-cell responses to both allogeneic lymphocytes and hapten-modified self are impaired, and Hurtenbach and Shearer (1982) found the latter to be controlled by an induced T-suppressor cell. It is of interest in this regard then, that Anderson et al (1984) could not detect any MHC transplantation antigens on post-meiotic germ cells.

The lower temperature of the testis has also been proposed as a possible factor conveying immune privilege (see Bobzien et al, 1983) although Selawry and Whittington (1984) reported that the lower temperature of the testis may in fact be detrimental for the survival and function of some grafts.

Seminal plasma from several species has been shown to suppress the in vitro stimulation of T and B lymphocytes (Stites and Erickson, 1975; Prakash et al, 1976; Lord et al, 1977; Marcus et al, 1978; Anderson and Tarter, 1982; Peluso et al, 1986). Tarter and Alexander (1984) have reported seminal plasma samples of primarily prostatic, vesicular, or epididymal/testicular origin as well as samples from vasectomised men to all contain complement-inhibiting activity, although epididymal fluid contained nearly twice the amount as other sources. That samples from vasectomised men showed the same level of inhibition suggests that secretion comes from the prostate and seminal vesicles, as well as from the testis and epididymis. However the testicular contribution may be important in maintaining the immunological barrier provided by the Sertoli cells for the developing germ cells, and Tarter and Alexander (1984) cite evidence of a high molecular weight anti-haemolytic factor secreted by Sertoli cells in tissue culture. Secretion of such factors into the interstitial region might be of consequence for immune function in this region.

Finally, the evidence that culture of tissue prior to transplantation will greatly increase the chances of graft survival is of interest. Lafferty (1980) and Hart and Fabre (1981) have demonstrated that passenger leukocytes, and particularly dendritic cells with abundant Ia antigen, contribute significantly to the immunogenicity of allografts. The process of culture removes such cells, and diminishes the effect. Thus, while some specific process might operate locally in the testis to provide immunosuppression, one might envisage the testis as providing a culture-like environment where the gradual replacement of donor passenger cells by host cells would significantly weaken the capacity of a graft to evoke a rejection response. This might in fact allow induction of tolerance in the hosts immune system, with the continued presence of low amounts of donor antigen.

1.4. THE PURPOSE OF THIS STUDY

The aim of the present study was to re-examine the immunologically-privileged status of the testis. Initially, work was carried out in rats to confirm the privileged status of the rodent testis. Once a routine transplantation protocol was established, various aspects of testicular function were modified experimentally to examine those aspects of potential importance for allograft survival in this site.

All previous work in this area has utilised rodents and related species. These animals have a testicular interstitial anatomy that is different to that of larger mammals. To allow investigation of the immune state of a testis more representative of the larger mammals,

this study was extended to include the sheep as an experimental model.

Following these studies on graft survival, a number of experiments were conducted (primarily in rats) with the aim of elucidating factors in the interstitial milieu of the testis that might contribute to the immune privilege afforded this region. Existing techniques for sampling interstitial fluid were found to be generally unsatisfactory, and potentially unphysiological, and a new technique involving the use of a Push-Pull cannula was tested. A number of experiments were performed where the push-pull cannula was used to sample interstitial fluid from rat testes in which normal function was altered by various experimental manipulations. The technique was also tested in sheep and pigs. The results of these studies have led to the development of a new hypothesis concerning physiological aspects of hormone secretion by the endocrine testis.

The opportunity to collaborate with Professor Mikko Niemi in Turku, Finland, at this time allowed a study of the immunocompetent cells in the testis of the rat and the ram. This revealed potentially important aspects of immune function in the testis that might contribute to an immunologically privileged environment, and the present study concludes with a new proposal as to why the rodent testis is an immunologically privileged site.

CHAPTER 2: MATERIALS AND METHODS

2.1. REAGENTS

LHRH, TRH, L-Thyroxine, Human Chorionic Gonadotrophin and Testosterone were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. TRH for injection was obtained from Roche Diagnostica (Protirelin). Radiolabelled ^{3}H-Testosterone, ^{125}I-L-Thyroxine, Sodium ^{125}Iodide, and Sodium ^{131}Iodide (Injection BP) were purchased from Amersham (Australia) Pty Ltd, Sydney, N.S.W. Other materials were obtained as follows: Heparin from Commonwealth Serum Laboratories, Melbourne, Victoria; Sodium pentobarbitone (Nembutal, Abbott) from Ceva Chemicals Australia Pty. Ltd., Hornsby, N.S.W.; polyethelene and polyvinylchloride cannulae tubing from Dural Plastics and Engineering, Dural, N.S.W.. All solvents used were analytical grade. Tissue culture media were obtained from Gibco Laboratories, Grand Island, New York, and from the Commonwealth Serum Laboratories, Parkville, Melbourne, Victoria.

2.2 ANIMALS

Rats were of the Porton strain bred in the Adelaide University central animal house and were not inbred; sheep used were of the Merino strain, bred on the university's Mortlock experiment station and were also not inbred.

2.3. SURGICAL PROCEDURES

2.3.1. Thyroidectomy

Rat

The paired thyroid glands are small pink organs, one on either side of the trachea just below the larynx. They are connected across the ventral aspect of the trachea by a thin band of tissue, the isthmus. Because the minute parathyroid glands are embedded in the anterior part of each thyroid gland and cannot be satisfactorily removed from them, the removal of the thyroid is technically a thyro-parathyroidectomy.

Each animal was anaesthetised with an intraperitoneal injection of pentobarbitone (60 mg/kg bw; Nembutal, Abbott) and laid on its back with its head toward the investigator. A midline skin incision was made along the length of the neck from its base to just below the point of the lower jaw. The subcutaneous fat and connective tissue were cleared and the salivary glands separated and retracted laterally. The two halves of the sternohyoid muscle were also separated and retracted laterally, using a paperclip reformed for this purpose. The thyrohyoid muscle running along the dorso-lateral aspect of each lobe was separated from each gland with blunt dissection and retracted along with the sternohyoid muscle. The isthmus was then cut in the midline and the connective tissue between each gland and the trachea carefully cleared by repeatedly pushing the gland away from the trachea. Extreme caution is needed near the dorsal edge of the gland since it runs along the recurrent laryngeal nerve. Severing of this nerve can cause paralysis of the larynx leading to respiratory

impairment which can be fatal (Waynforth, 1980). Each gland was finally severed from its vascular connections anteriorly and posteriorly and removed.

Sheep

The thyroid of the sheep is a reddish-brown colour and is situated on the lateral and ventral surfaces of the cranial end of the trachea (May, 1964). As in the rat, it consists of two lateral lobes united at their caudal ends by the fibrous or glandular isthmus which passes across the ventral surface of the trachea. The lateral lobes extend from the first or second tracheal ring to the tenth or eleventh ring and measure 4 to 5 cm long and 1 to 1.5 cm wide. They are smooth, elongate and oval in outline. The gland is loosely attached to the trachea and is covered laterally and ventrally by the sternocephalic, omohyoid and sternothyroid muscles. One pair of parathyroids are situated on the deep face of each lobe near the caudal border and are inevitably removed during thyroidectomy. However, ruminants also possess a pair of external parathyroids found cranially to the thyroid gland, and thus thyroidectomy in sheep does not also involve total parathyroidectomy, as in the rat.

For thyroidectomy, each animal was fasted for 48 hours before surgery, and kept off water for 24 hours before surgery to reduce fluidity of rumen contents. This is important in sheep, because during the operation the animal is placed on its back and the head is sometimes positioned lower than the rumen, causing rumen contents to flow back up the oesophagus during anaesthesia. The likelihood of this can be greatly reduced by the above preventative measures. Anaesthesia was induced by an intravenous injection of pentobarbitone (60 mg/kg

bw) into the jugular and maintained by pentobarbitone administered as needed through a cannula in the lateral saphenous vein. To insert this cannula, a skin incision was made in one of the rear legs, laterally, above the tarsal joint at the distal end of the tibia. Blunt dissection through the fascia revealed the lateral saphenous vein (recurrent tarsal vein, see May, 1964). A section of this vein was cleaned of connective tissue on all sides and two silk threads placed around the vessel. The first thread was securely tied proximal to the proposed point of insertion, a small cut was made in the wall of the vein above the first tie and a polyethylene cannula (O.D. 1.5mm, I.D. 1.0 mm) introduced into the vein for a distance of 30-40 cm. The second thread was then tied securely around the vein and the enclosed cannula above the insertion point, to secure the cannula in position.

A mid-ventral incision was made in the anterior part of the neck starting just behind the cricoid cartilage. The subcutaneous facia was separated by blunt dissection and the muscles covering the gland were separated at the midline and retracted laterally. The thyroid in the sheep is exceptionally well vascularised, with blood supplied from paired posterior and anterior thyroid arteries which arise from the carotid artery. Venous drainage is mainly through the paired anterior thyroid veins which join the jugular vein. Each lobe was carefully separated from the surrounding connective tissue and the trachea, and all blood vessels ligated prior to removal of both lobes and the isthmus. Minor bleeding was controlled with gauze swabs. Before closing the skin incision, retractors and swabs were removed and muscles allowed to return to their normal positions. As in the rat, no suturing of the muscles was required.

2.3.2. Hypophysectomy

The pituitary gland is a small pink organ found at the base of the brain. It is located in a small bony fossa, the sella turcica, which is in the sphenoid bone of the cranium. The pituitary is attached to the floor of the third ventricle by a thin stalk and is in close relationship with the optic chiasma.

Rats

Rats were hypophysectomised by Mr J.L. Zupp using the intra-aural method of Gay (1967). The special stereotaxic apparatus required to perform this operation successfully was obtained from the Stoelting Company, Chicago, USA. Each animal was anaesthetised by an intraperitoneal injection of Avertin (0.8 ml/100 g bw), which is no longer available commercially but can be constituted from a solution of tribromoethanol and amylene hydrate as described by Dyer et al (1981). This relatively short acting anaesthetic was found preferable to pentobarbitone. The animal was placed on its ventral surface with its tail away from the operator. The cartilage at the base of the external auditary meatus was cut with a pair of scissors, the animals teeth placed into the tooth holder, and the ear bars inserted into the ears. An 18G thin-walled needle mounted on a 10 ml syringe containing 2-3 mls of physiological saline was inserted into the hole in the ear bars. The needle was pushed along the ear canal at an angle of 5^{o} to the horizontal in a dorsal direction until it touched the medial wall of the periotic capsule where the bone is particularly thin. The needle was then forced through the bone with the bevel facing upwards so that the point slid under the trigeminal nerve without damaging it.

The needle was then pushed forward a predetermined distance (established in sacrificed animals of the same size, with the brain removed) so that the bevel could be turned down to be situated directly over the pituitary, under the sella turcica. The gland was then slowly aspirated into the water-filled syringe and carefully examined to ensure complete removal. Post-operative care is vitally important to ensure a low mortality rate from hypophysectomy. Animals were kept warm after the operation, and given a 5% dextrose solution ad libitum to prevent development of fatal hypoglycaemia. This was routinely combined with a Terramycin solution (Pfizer Agricare Pty Ltd, West Ryde, NSW) to reduce infections.

To obtain tissue for transplantation purposes, donor animals were sacrificed using an overdose of chloroform. The skin on top of the skull was removed, and the skull scraped free of connective tissue and fat. Using a dental drill with a round bone burr, the top of the skull was scored,cut and lifted off with the dura. Maintaining sterility, the brain was removed to allow access to the sella turcica. The area was carefully cleaned and using sterile fine forceps, the sella was lifted to expose the pituitary. This was then carefully removed and placed into sterile Dulbecco's Phosphate Buffered Saline (Gibco, Grand Island, New York) ready for transplantation.

Sheep

The pituitary is an irregular conical shaped structure in sheep. It is slightly reddish-brown and is relatively larger in sheep than in other domestic animals (May, 1964). Pituitaries were removed for transplantation from sacrificed Merino males castrated soon after birth (Wethers). These animals were decapitated, and the top of the

skull removed with an electric band-saw. As in rats, sterility was carefully maintained. The brain was removed to expose the sella turcica which was carefully lifted with sterile forceps. The pituitary was then carefully removed and placed into sterile Dulbecco's PBS ready for transplantation.

2.3.3. Cryptorchidectomy

Each animal was anaesthetised with pentobarbitone (see 2.3.1.) and laid on its back with the tail towards the investigator. A small median incision was made in the skin of scrotum, to visualise each testis in its muscular sac. Each sac was cut open, and each testis pushed up through the inguinal opening into the abdominal cavity. The inguinal canal on each side was then sutured closed.

2.3.4. Efferent Duct Ligation

To tie the efferent ducts in rats, each animal was anaesthetised with pentobarbitone (see 2.3.1.) and laid on its back with the tail towards the investigator. A testis was exposed through an abdominal incision, and the head of the epididymis lifted from the testis by carefully dissecting the joining connective tissue on the lateral side. This allowed the epididymis to be rolled over to one side to expose the efferent ducts. These were dissected free from the surrounding fat and occluded by tying one ligature (4/0 silk, Cyanamid, Australia) distally along the efferent ducts to the testis, and another ligature around the ducts very close to the testis. The testis was then returned to the scrotum, the abdominal incision

closed, and the animal allowed to recover.

2.3.5. Blood Collection

2.3.5.1. Rats

Peripheral blood samples were taken from the jugular vein in recovery animals and the posterior vena cava in non-recovery animals. For jugular samples, the animal was anaesthetised with pentobarbitone (see 2.3.1.) and laid on its back with its head towards the investigator. The base of the neck was opened and the jugular vein exposed through the pectoral muscle. A 21G needle attatched to a 1ml syringe was introduced into the vein, and blood withdrawn slowly. After removal of the needle, the skin incision was sutured. In non-recovery animals, after anaesthesia was induced with pentobarbitone, the animal was laid on its back with its tail towards the investigator. A V-cut was made through the skin and abdominal wall starting at the base of the abdomen and proceeding diagonally across each side to end up at the dorso-lateral edges of the thorax. The flap of skin was moved onto the chest wall and the entire gut reflected. The vena cava was entered at the widest point (near the level of the kidneys) using a 21G needle attatched to a 2ml syringe, and blood withdrawn slowly. In both situations, blood samples were transferred to heparinised Eppendorf tubes, and plasma separated by centrifugation in a Beckman Microfuge.

Testicular venous blood samples were collected from the veins on the surface of the testis. After exposing the testis, usually via a scrotal incision, the point was identified at which the testicular

veins merged on the surface of the testis prior to separating again to form the pampiniform plexus. Using a 27G needle, the largest vein was punctured at the point of minimun branching. The needle was removed, and heparinised microhaematocrit tubes (Capillets, Dade Diagnostics, Puerto Rico) were held against the resultant pool of blood flowing from the vein. In most cases, ten such tubes were collected from each testis. The contents of these tubes were then transferred to a plastic Eppendorf tube and centrifuged to separate the plasma in a Beckman Microfuge.

In all cases, plasma was stored at -20 ^{o}C until assayed.

2.3.5.2. Sheep

Peripheral blood samples were taken in conscious animals from the jugular by venipuncture, using a 19G needle attached to a 10ml syringe. In those experiments where repetitive sampling was required in conscious animals, the jugular was cannulated with an indwelling polyethylene cannula (I.D. 1.00 mm, O.D. 1.5 mm) on the day prior to sampling. In anaesthetised animals, peripheral blood samples were taken from the posterior vena cava via a cannula positioned in a lateral saphenous vein as described in 2.3.1.

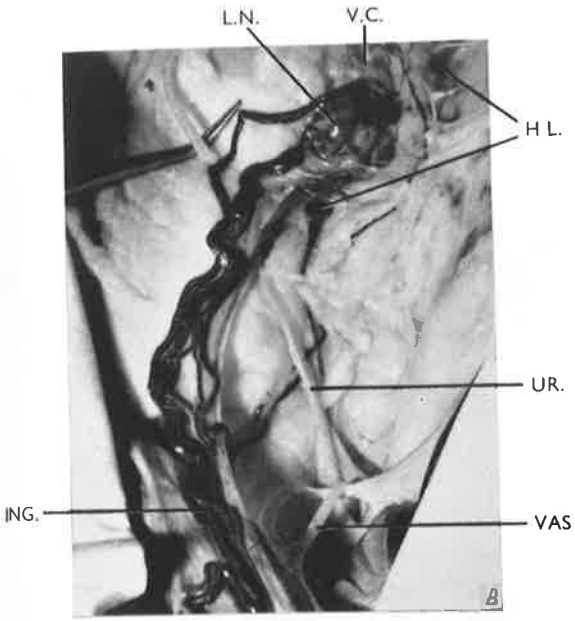
Testicular venous blood was obtained from anaesthetised animals in which an indwelling polyvinylchloride cannula (I.D. 0.8 mm, O.D. 1.2 mm) was positioned in the ipsilateral internal spermatic vein (see Figure 2.1.).

After drawing a blood sample the cannulae were flushed with 0.9% sterile saline containing heparin (50 I.U./ml). Blood samples were collected into 10 ml heparinized centrifuge tubes and usually

Figure 2.1. Lymphatics draining the testis in the ram. The animal was killed 15 minutes after receiving an injection of indian ink into the testis. The course of the lymph (black) from the testis to the lumbar lymph node (L.N.) is shown. The intestines have been removed. The deep inguinal node (D.I.N.) remained unstained (from Lindner, 1963).

A. Exposure of testicular lymphatics in the spermatic cord. L, lymph vessels containing indian ink; PL, pampiniform plexus drained of blood; CR, cremaster muscle; T.V., tunica vaginalis, reflected; VAS, vas deferens, retracted; X, level at which lymphatics and internal spermatic veins were cannulated; I.R., external inguinal ring.

B. Testicular lymphatics in inguinal canal and abdomen. ING., inguinal canal, exposed; VAS, vas deferens curving around ureter (UR); L.N., lumbar lymph node; V.C., posterior vena cava; H.L., lumbar haemolymph nodes.


L.N.
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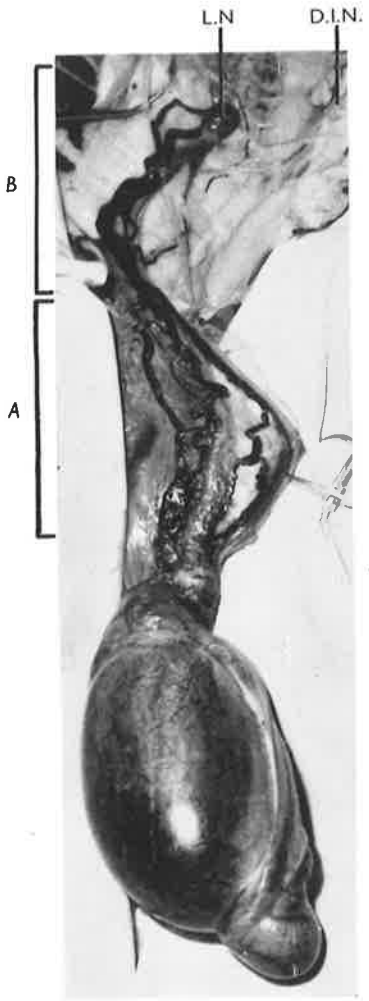
L.N
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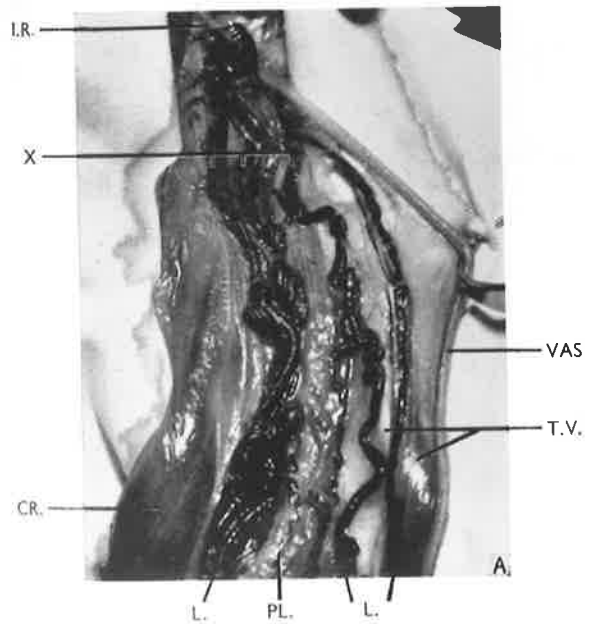

I.R.
X
VAS
T.V.
CR.
A
L.
PL.
L.

centrifuged immediately. If there was an appreciable time lag between collection and centrifugation the samples were kept on ice. The plasma separated after centrifugation were stored at -20°C, until required for assay.

2.3.6. Lymph Collection

Testicular lymph was obtained from anaesthetised animals in which a polyvinylchloride cannula (I.D. 0.8mm, O.D. 1.2 mm) was positioned in a lymphatic vessel in the spermatic cord (Lindner, 1963, 1967; Cowie et al, 1964; see Figure 2.1.). It was important to have this cannula loaded with heparinised saline at the time of insertion, and to ensure that lymph flowed freely after insertion.

2.3.7. Rat Transplants

2.3.7.1. Under the Kidney capsule

Animals were anaesthetised with pentobarbitone (see 2.3.1.) and shaved on the flank over the kidney destined to receive the tissue graft. An incision was made in the flank and the kidney exposed by gently isolating it from the surrounding fat. Using a sharp pair of forceps and maintaining sterility, a small hole was made in the kidney capsule and with the aid of fine toothed forceps, the tissue graft was placed beneath the capsule. Great care was taken not to damage the kidney itself. The organ was then returned to the abdomen and carefully repositioned in the fat cover. The incision was then closed.

2.3.7.2 Into the Testis

Animals were anaesthetised with pentobarbitone (see 2.3.1) and shaved on the abdomen. A midline incision was made in the abdomen, and the testis gently pushed up from the scrotum into the abdomen, to be exposed through the incision.

Whole tissue grafts

When transplanting a piece of tissue, a 25G needle was used to puncture the tunica and fine toothed forceps used to insert the tissue graft into the testicular parenchyma. With this technique, suturing of the tunica was rarely needed.

Isolated cells

If the graft consisted of isolated cells, these were loaded into a 1 ml syringe in a small volume of carrier buffer, and using a scalp-vein needle set, the tunica was punctured and the needle of the scalp-vein set pushed into the testicular parenchyma and the cells gently expelled. The scalp-vein needle was then withdrawn. As with whole tissue grafts, suturing of the tunica was rarely required.

After transplantation, the testis was carefully returned to the scrotum ensuring that the spermatic cord was not twisted in the process. The abdominal incision was then closed.

2.3.8. Transplants into the Ram Testis

The ovine testis contains much more connective tissue than the rodent testis and tissue grafts cannot be simply pushed into the testicular parenchyma. Consequently, a different method for transplantation had to be developed. The tissue to be transplanted was carefully cut into pieces of approximately 1 mm^3. The testis was partly exposed through a small incision in the scrotum and a purse-string suture of approximately 1 cm diameter placed in the tunica in a region devoid of any obvious blood vessels. The diced tissue was then loaded into a 12-gauge stainless steel cannula which was pushed into the testis well clear of the rete testis, through the centre of the purse-string suture. A trocar was then used gently to expel the tissue into the testis. As the cannula was removed from the testis, the purse-string suture was pulled closed. The scrotal incision was then sutured, and the animals returned to their pens after recovery.

2.4. ISOLATION AND CULTURE OF RAT PITUITARY CELLS

2.4.1. Buffers and Solutions

Hepes buffer

137 mM	NaCl
5 mM	KCl
0.7 mM	Na_2HPO_4
25 mM	n-2-hydroxyethyl piperazine ethanesulfonic acid (HEPES), pH 7.2

10 mM Glucose

360 uM $CaCl_2$

Dulbeccos modified Eagles medium (DMEM)

This buffer was obtained in powdered form, specified suitable for tissue culture, from Gibco Laboratories, Grand Island, New York, and reconstituted according to the directions of the supplier.

Krebbs Ringer Bicarbonate Solution with Albumin (KRB/BSA)

Solution 1. 0.154 M NaCl

Solution 2. 0.154 M KCl

Solution 3. 0.11 M $CaCl_2$

Solution 4. 0.154 M KH_2PO_4

Solution 5. 0.154 M $MgSO_4.7H_2O$

[Solutions 1 to 5 prepared as stocks of 5X given concentration]

Solution 6. 0.154 M $NaHCO_3$ (gassed for 1 hour with CO_2)

Krebbs Ringer Bicarbonate (KRB)

100 parts solution 1

4 parts solution 2

3 parts solution 3

1 part solution 4

1 part solution 5

21 parts solution 6

Gassed for 10 minutes with 5 % CO_2/95 % O_2

To this KRB solution was added

14 mM Glucose

3 mg/ml Bovine serum albumin

Complete amino acid supplement (Eagle, 1959)

KRB/BSA (Ca^{++}-Mg^{++} free)

Prepared as for KRB/BSA above, with solutions 3 and 5 omitted.

2.4.2. Collagenase digestion (after Vale et al, 1972)

Anterior pituitary glands were obtained as detailed in 2.3.2. and rinsed several times in Hepes buffer. Pituitary quarters were incubated in Hepes buffer containing 3% BSA, 0.1% hyaluronidase and 0.35% collagenase, at 37°C for 45 minutes with continual stirring. Fragments of pituitary were drawn in and out of a siliconised Pasteur pipette every 10 minutes during this incubation. The incubation mixture was then centrifuged at 475 g for 2 minutes, the cellular pellet resuspended in Hepes buffer containing 0.25% Viokase and stirred for another 10 minutes. Following a second centrifugation, pelleted cells were resuspended in sterile DMEM medium containing 10% horse serum, 2.5% fetal calf serum, 0.1 mM glutamine and 1% Gibco non-essential amino acids. Cells were washed 6 times in sterile medium by repeated vortexing and centrifugation. After the final wash, cells were suspended in a small volume of sterile DMEM for transplantation

as described in 2.3.7.2.

2.4.3. Trypsin digestion (after Hopkins and Farquar, 1973)

Anterior pituitary glands were obtained as described in 2.3.2. and each anterior lobe cleanly chopped with a razor blade into 40-50 rectangular tissue blocks. Blocks were placed into KRB/BSA at room temperature during collection and finally washed once with KRB/BSA. All blocks were then pooled into an Erlenmyer flask with 5 ml KRB/BSA containing 12.5% trypsin and 20 ug/ml DNAase at 25 ^{o}C, and transferred to a shaking water bath for incubation at 37 ^{o}C for 15 minutes. After trypsin incubation, the solution was transferred to a conical centrifuge tube, the supernatant removed after settling of tissue contents (about 1 minute) and 5 ml of pre-warmed KRB/BSA containing 1 mg/ml soya bean trypsin inhibitor added. This was then incubated for 5 minutes at 37 ^{o}C. The supernatant was again decanted, and the tissue washed with Ca^{++}/Mg^{++} free KRB/BSA containing 2 mM EDTA and incubated for 5 minutes at 37 ^{o}C. After decanting this solution, 4 ml of Ca^{++}/Mg^{++} free KRB/BSA containing 1 mM EDTA and 8 ug/ml neuramindase was added. The solution was transferred to another Erlenmyer flask and incubated in the shaking water bath at 37 ^{o}C for 15 minutes. The tissue was then transferred to a conical centrifuge tube and washed 3 times with 20 ml of Ca^{++}/Mg^{++} free KRB/BSA. Rigorous pipetting of the mixture with a flame-polished Pasteur pipette was then used to disperse the blocks of tissue. Finally, the cell suspension was spun through BSA by layering 5 ml of 4% BSA below the cell suspension. The tube was centrifuged at 70 g for 10 minutes to produce a cell pellet relatively free of debris, which was then resuspended in a small

volume of DMEM for transplantation as described in 2.3.7.2.

2.4.4. Tissue Culture

2.4.4.1. Routine method

Following isolation of pituitary cells as described in 2.4.3. above, 2.5 X 10^5 cells were placed into each of 4 wells on a tissue culture plate (Costar, Cambridge, Mass., USA). 0.5 ml of DMEM containing 5% horse serum, 2.5% fetal calf serum and 1% non-essential amino acids was then added to each well. Cells were maintained under an atmosphere of 5% CO_2 in air in an incubator at 37 ^{o}C. Culture medium was changed every second day during incubation.

2.4.4.2. LHRH Challenge

Five days after plating out of isolated pituitary cells, their functional capacity was assessed by providing a stimulus of LHRH. Cells were washed several times with medium as detailed in 2.4.4.1. and then finally given serum-free medium containing only 1% non-essential amino acids. In each test for a 4 well culture, 2 wells received only serum-free medium and 2 wells received serum-free medium containing 10 nM LHRH. Cells were incubated for 6 hours with these solutions, after which the medium was sampled from around the cells and stored at -20 ^{o}C until assayed for LH. Cultures were terminated at this point.

2.5. THYROID FUNCTION TEST

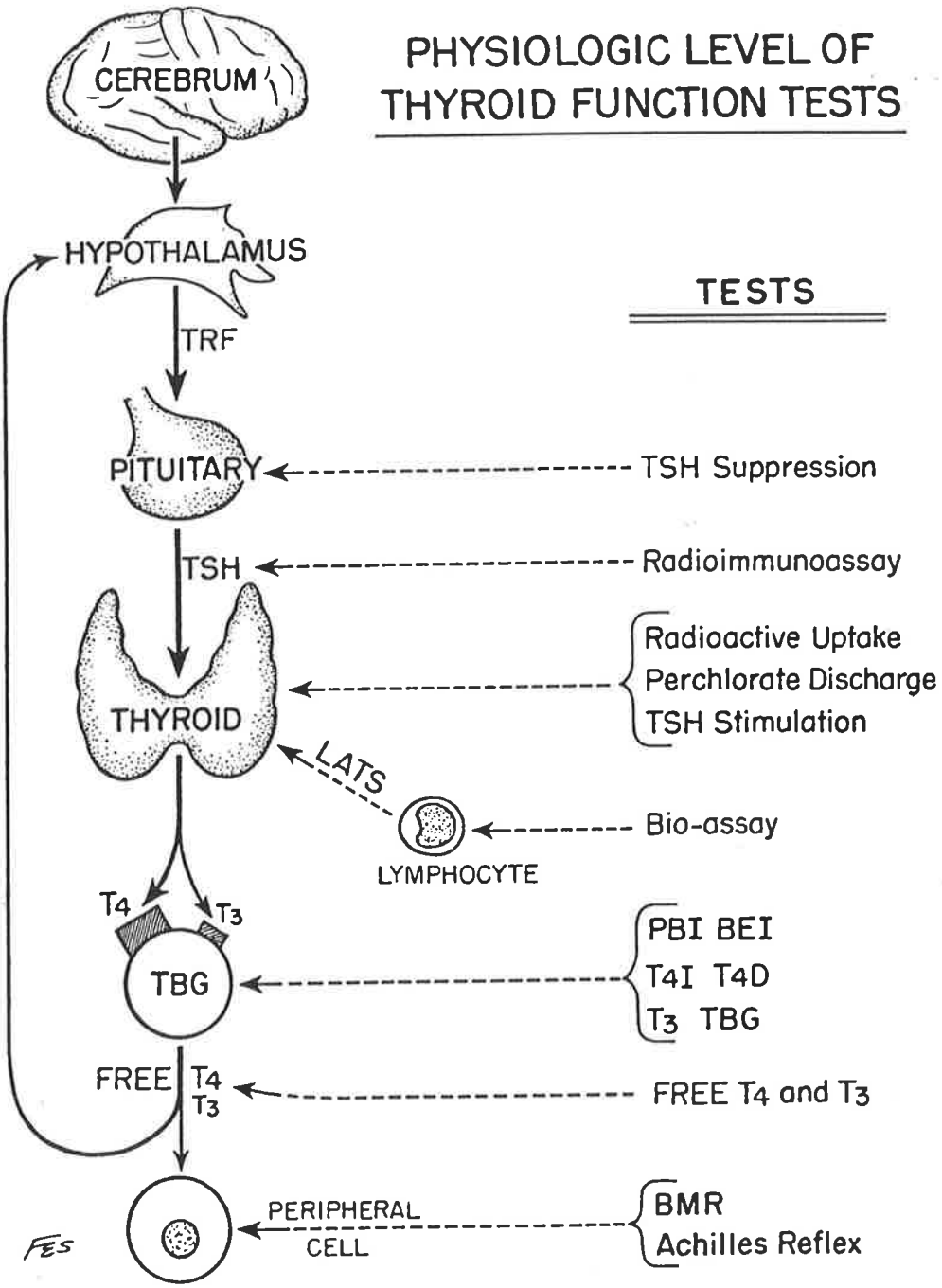
A variety of tests exist to assess the function of the thyroid gland, as seen in Figure 2.2. Three methods were found to be of use in the studies in this thesis. Firstly, in some experiments it was useful to monitor plasma levels of T_4 and TSH, and these methods are detailed in 2.13.2. and 2.13.3.7. In other experiments, it was possible to provide a TRH bolus to stimulate TSH secretion by the animals' own pituitary which in turn should stimulate T_4 secretion by the graft. This could then be monitored in plasma samples. It was not possible to directly challenge the grafts with oTSH because this was obtained from the USA and quarantine regulations would not permit its use in vivo. The TRH on the other hand was a synthetic analog and did not have such restrictions on its use. Finally, in most experiments the ability of a graft to accumulate radioactive iodine was assessed.

2.5.1. Iodine Accumulation

2.5.1.1. Rats

Twenty-four hours prior to graft removal, rats were given an intraperitoneal injection of 1.0 uCi (37 kBq) ^{125}Iodine. Following removal of the testis or kidney containing the graft, and the contralateral ungrafted organ, testes were placed into Bouins fixative and kidneys into buffered Formalin (see 2.8.). Uptake of iodine in all tissue samples was monitored in a gamma counter (Riagamma 1271, LKB-Wallac, Finland).

Figure 2.2. Physiologic level of thyroid function tests (from Zellmann, 1974).


PHYSIOLOGIC LEVEL OF
THYROID FUNCTION TESTS
CEREBRUM
HYPOTHALAMUS
TRF
PITUITARY
TSH
THYROID
LATS
LYMPHOCYTE
T4
T3
TBG
FREE T4
T3
PERIPHERAL
CELL
FES
TESTS
TSH Suppression
Radioimmunoassay
Radioactive Uptake
Perchlorate Discharge
TSH Stimulation
Bio-assay
PBI BEI
T4I T4D
T3 TBG
FREE T4 and T3
BMR
Achilles Reflex

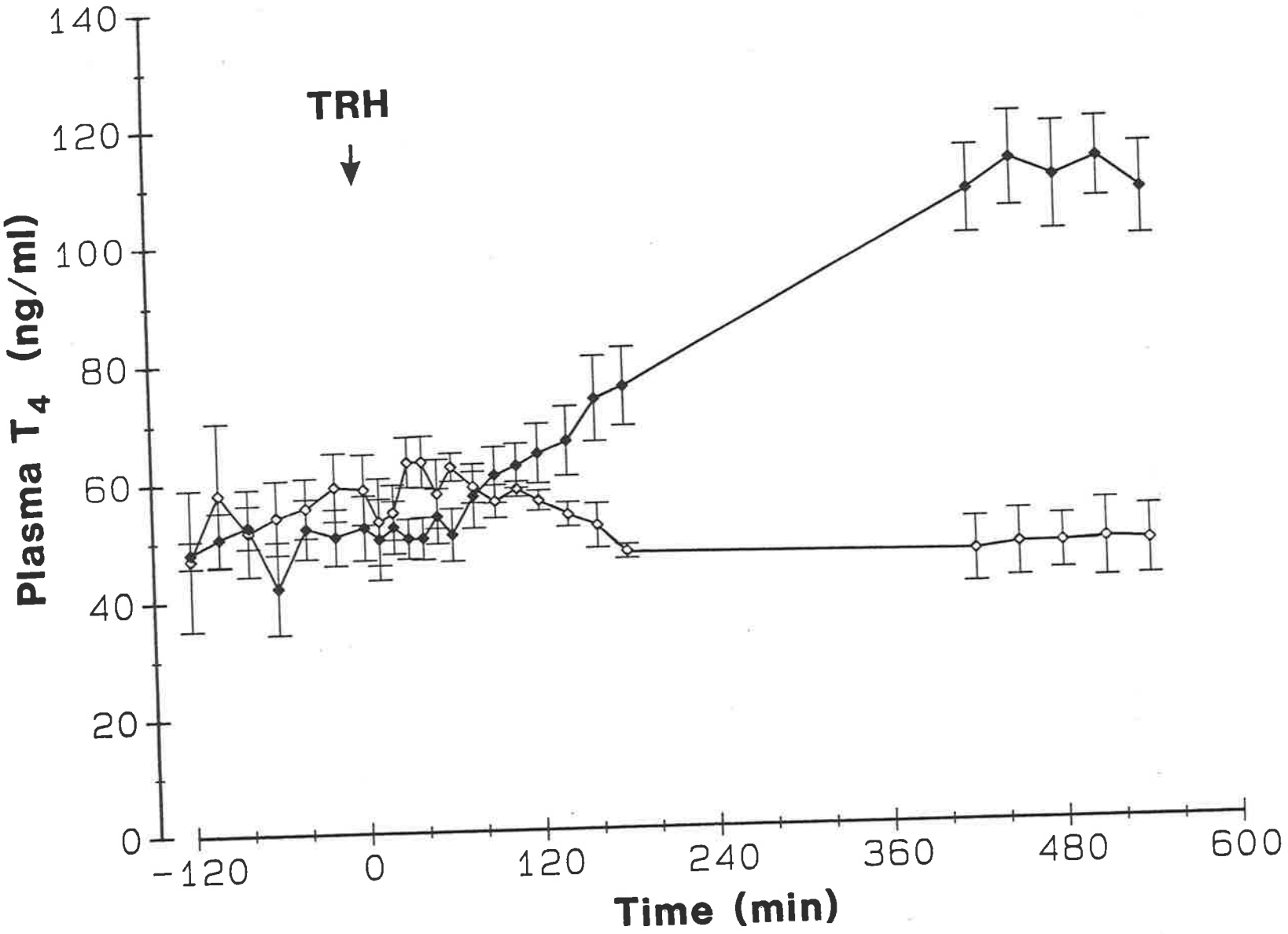
2.5.1.2. Sheep

Iodine localisation by thyroid grafts in sheep testes was assessed initially by gamma photography in the Dept. of Radiology at the Flinders Medical Centre, Bedford Park, South Australia. To aid in photography, ^{131}Iodine was used instead of ^{125}Iodine. Each animal was given an intravenous bolus of 1 mCi ^{131}I-injectable solution (Amersham, Australia) 48 hours prior to graft removal. Following castration, testes were placed on ice and transferred to the Flinders Medical Centre for gamma photography. Upon return to the laboratory, each testis was serially cut into 1 cm^3 pieces which were placed into Bouins fixative and counted in a gamma counter.

2.5.2. TRH Challenge

Sheep were fitted with indwelling polyethylene jugular catheters (1.0 mm o.d, 1.5 mm i.d.). A bolus injection of 200 ug TRH was administered intravenously (i.v.) in 2 ml physiological saline (0.9% NaCl) to all animals. This dose was selected from a dose-respose trial as being able to induce satisfactory Thyroid Stimulating Hormone (TSH) secretion and associated Thyroxine (T_4) release in normal rams (see Figure 2.3.). Bleeding commenced two hours prior to TRH administration at 20 min intervals and continued for 3 hours after TRH administration at 10 minute intervals for the first hour, and every 15 minutes for the next two hours. Further samples were collected at 30 minute intervals starting 7 hours after TRH administration and continuing for another 2 hours. Samples were centrifuged and plasma was stored at -20^{o}C until assayed for TSH and T_4.

Figure 2.3. Levels of Thyroxine in peripheral plasma of rams after an intravenous injection of 2ml physiological saline (open diamonds) or 200 ug TRH in 2 ml saline (solid diamonds).


TRH
Plasma T_4 (ng/ml)
140
120
100
80
60
40
20
0
-120
0
120
240
360
480
600
Time (min)

2.6. PITUITARY FUNCTION TEST - LHRH CHALLENGE

2.6.1 Intravenous injection

Pituitary function in sheep was assessed by monitoring the response of the pituitary to a bolus injection of LHRH. To ensure that the donor pituitaries were functioning normally prior to transplantation, donor wethers were prepared with indwelling jugular catheters as described in 2.3.5.2. and challenged with an intravenous bolus of LHRH (5ng/kg body weight) injected into the uncannulated jugular vein. Blood samples were taken 20 min prior to the LHRH being administered, and continued for two hours after administration at 10 min intervals to monitor LH secretion.

2.6.2. Intravenous infusion

2.6.2.1. Validation of method

Prior to transplantation, rams that were to be used as graft recipients were infused with LHRH via a cannula in the lateral saphenous vein in the hind leg (see 2.3.1.) to check that an amount of LHRH which, if infused into the testicular artery to provide sufficient local concentrations of LHRH to stimulate gonadotrophin secretion by viable intratesticular pituitary transplants, would not be sufficient in the peripheral circulation to stimulate the recipient animals' own pituitary into secreting gonadotrophins. The jugular vein in these animals was cannulated with an indwelling PE catheter as described in 2.3.5.2. Samples were collected 20 min prior to

commencement of the infusion, and then continued at 10 minute intervals for two hours. The infusion itself was maintained for one hour.

2.6.2.2. Calculation of dose of infused LHRH

Intravenous injection of LHRH at 5 ng/kg body weight (bw) produces a physiological pulse of LH in wethers (D'Occhio et al, 1982) and the calculated half-life for LHRH in sheep ranges from 5 to 10 minutes (Swift and Crighton, 1979). However for the infusion of LHRH into the testis, calculations were based on double the normal intravenous dose of LHRH (increased to 10ng/kg bw) to ensure that sufficient hormone reached the grafts. Assuming an extracellular fluid volume of 15% bw, an i.v. bolus injection of 10ng LHRH/kg bw into the jugular of a 60kg animal would produce concentrations at the level of the pituitary in the order of 70 pg/ml. To produce a similar concentration in a testis with an assumed blood flow of 30 ml/min (see Setchell and Brooks, 1987) would require an LHRH infusion of 2 ng/min into the testicular artery and this rate was chosen for infusing grafted testes. The total amount of LHRH infused directly into the testicular artery was 120 ng, or about 2 ng/kg bw. This is hardly sufficient to stimulate the recipient's own pituitary, even if given as a single bolus, and was expected to be quite ineffective in this regard when infused over 1 hour.

2.6.2.3. Intratesticular arterial infusion

To examine graft function before the testis was removed, an

expanded catheter (PE, nominal I.D. at finer end 0.2mm, O.D. 0.5mm) was inserted into the testicular artery on the surface of the testis. LHRH (20 ng/ml) was infused through this catheter at the rate of 0.1 ml/min (final dose of 2 ng/min as calculated in 2.6.2.2.) for one hour to stimulate gonadotrophin secretion by the grafts.

2.7. HCG CHALLENGE

HCG was administered to rats as a subcutaneous injection of 50 i.u. hCG (Sigma, St. Louis) in 0.2 ml 0.01 M phosphate buffer (pH 7.2).

2.8. EDS ADMINISTRATION

Ethylene dimethane sulphonate (EDS) was kindly provided by Dr F.F.G. Rommerts of Erasmus University, Rotterdam, The Netherlands. EDS (30 mg/ml) was dissolved in dimethyl sulphoxide water (1:3, vol/vol) and administered via intraperitoneal injection (100 mg/kg bw).

2.9. SILICA ADMINISTRATION

Silica (SiO_2, Sigma, particle size 1-5 u; 5 mg in 0.2 ml physiological saline) was administered by intratesticular injection using a 25G needle and 1 ml syringe.

2.10. MEASUREMENT OF ALBUMIN AND Cr-EDTA DISTRIBUTION VOLUMES

One-hour ^{51}Cr-labelled ethylene diamine tetra-acetic acid

(^{51}Cr-EDTA) and ^{125}I-labelled human serum albumin (^{125}I-hSA) 'spaces' in the testis were measured using the method described previously by Setchell and Sharpe (1981). Animals were anaesthetised with pentobarbitone sodium (Nembutal, Abbott, 60 mg/kg intraperitoneally). A polyvinylchloride catheter (0.8mm o.d., 0.5 mm i.d.) was inserted into one jugular vein, and for albumin space determinations 1 uCi of ^{125}I-hSA (Amersham, Australia) was injected through the catheter. For Cr-EDTA space measurements, both ureters were severed prior to injection of 5 uCi of ^{51}Cr-EDTA (Amersham, Australia) through the catheter. One hour later, both testes were removed and a blood sample collected with a syringe and needle from the posterior vena cava (see 2.3.5.). Radioactivity in plasma and isolated testes was measured in a gamma spectrometer (LKB Wallac, Finland) using different settings to discriminate betweeen the two different isotopes used. The distribution volume or "space" for each marker in ul/g was calculated by dividing the radioactivity in the tissue in disintegrations/minute per gram by the radioactivity in the blood plasma in disintegrations/minute per ul plasma.

2.11. VASCULAR PERMEABILITY

Vascular permeability was calculated using the formula $PS = K. V_{alb\text{-}final}$ (Amtorp, 1980) where $V_{alb\text{-}final}$ is the final equilibrium volume of distribution of albumin (reached 6 hours after administration of ^{125}I-hSA, but found to equal the 1 hour Cr-EDTA space, Setchell and Sharpe, 1981; see 2.10.), K is the rate constant of penetration of albumin into the testis determined from the slope of the line $\log_e (1 - (V_{alb\text{-}1\ hour}/V_{Cr\text{-}EDTA}))$ and $V_{alb\text{-}1\ hour}$ and

$V_{Cr-EDTA}$ are the volumes of distribution of these markers one hour after their administration. In calculating the rate constant K, initial V_{alb} values (time = 0 min) were assumed to be equivalent to testis blood volume as described by Setchell and Sharpe (1981) and their values of 10 ul/g before hCG administration and 15 ul/g after hCG administration were used in calculations. In the equation described by Amtorp (1980) for calculation of vascular permeability, the logarithms were calculated to base e, although this was not specified by the original author. Unfortunately, in previous publications (see Setchell et al, 1984; Setchell and Zupp, 1987) values were calculated with base 10 logarithms. As a consequence, the absolute values for permeability-surface area products previously published are too low by a factor of 2.303, but this does not affect the validity of changes reported.

2.12. LYMPH FLOW

Lymph flow was calculated from the formula $Q_L = 0.693 \cdot V_{alb-final} / T_{1/2}$ where Q_L is the lymph flow in ul/g/min, $V_{alb-final}$ is the final equilibrium volume in ul/g of distribution of the albumin (assumed to equal the 1 hour Cr-EDTA space as discussed in 2.11.) and $T_{1/2}$ is the half-time of clearance of directly-injected albumin from the testis. For measurements of ^{125}I-hSA clearance rate from testes in-vivo, radioactivity was monitored using a directional gamma counter with a ratemeter (Nuclear Chicago, Illinois, U.S.A.) and a chart recorder (Rikadenki, Tokyo, Japan) following the injection directly into the testis of 1 uCi in 50 ul physiological saline, through a 30G needle.

2.13. HORMONE ASSAYS

2.13.1. Buffers and Solutions

2.13.1.1. Phosphate buffers

(A) 0.05 M, pH 7.5 (PBS)

1.42 g Na_2HPO_4 (anhydrous)

0.37 g EDTA

1.00 g NaN_3

8.18 g NaCl

adjusted to pH 7.5 with IN HCl

made up to 1 litre using double distilled water

(B) 0.05 M, pH 7.5, 0.2% gelatin (gel PBS)

To the PBS, added gelatin to get 0.2% solution.
adjusted pH to 7.5 with IN NaOH

(C) 0.07 M K_2HPO_4/KH_2PO_4, pH 7.4 (KPBS)

9.35 g K_2HPO_4

1.78 g KH_2PO_4

made up to 1 litre using double distilled water

2.13.1.2. Blocker solution with ^{125}I-thyroxine

0.0333 g 8-Anilino-naphthalene-sulphonic acid (ANSA)

1.3320 g Thimerosal (Merthiolate)

3.25 ml T_4 free serum (T_4FS)

0.25 ul ^{125}I-T_4 (specific activity: 40 - 60 uci/ug T_4)

made up to 100 ml with KPBS

2.13.1.3. T_4-free serum

To a pool of serum, sufficient ^{125}I-T_4 was added to give 30 - 40,000 CPM/ml. After mixing well, two 1 ml samples were taken for counting. 2 g charcoal/15 ml serum was then added and the solution left stirring overnight at room temperature. Following incubation, the solution was centrifuged for 30 mins at 2,000 rpm to remove the bulk of the charcoal, and then centrifuged at 20,000 rpm for 30 mins. This process was repeated until the counts in the supernatant serum were less than 1% of those added.

2.13.1.4. Liquid scintillation system

(A) Scintillation fluid

4.0 g PPO (2,5-diphenyloxazole)

0.4 g POPOP (2,2'-p-phenylenebis (5-phenyloxazole))

dissolved in 1 litre of toluene

(B) Counting of aqueous systems

After adding the aqueous sample and scintillation fluid, the polyethylene vials were shaken vigorously for 20 mins on a

mechanical shaker to facilitate the transfer of radioactivity from the aqueous to the organic phase. Radioactivity was determined in a LKB-Wallac, Rack Beta II liquid scintillation counter, Model No. 1215.

2.13.2. L-Thyroxine radioimmunoassay

Plasma thyroxine concentrations were determined using the antisera which was donated to B.F.Good, by C.W. Burke, Department of Endocrinology, Radcliffe Infirmary, Oxford, England. Validation of the assay has been described elsewhere (Burke and Shakespear, 1976).

Duplicate aliquots of plasma (10 to 50 ul) were added to disposable glass culture tubes (12 x 75 mm). A stock solution of 1 mg L-T_4 in 1 ml EtOH/NH_4OH (25 ml EtOH + 0.880 ml NH_4OH) was prepared. Standards of 0, 9.32, 18.64, 37.28, 62.1, 93.2, 186.4 ng/ml T_4 were prepared by diluting the stock solution in appropriate volumes of T_4FS. To 10 ul of standard or plasma was added 300ul of blocker solution containing ^{125}I-T_4 (approximately 10,000 CPM per 300 ul) (see Section 2.10.1.2.) and 200 ul of antisera (final dilution of 1:3000) and the mixture incubated for at least 12 hrs, at 4^{o}C. Free and bound T_4 were separated by the addition of 1 ml KPBS containing 8 mg each Dextran (Pharmacia Chemicals) and Charcoal. The tubes were shaken well and allowed to stand for 5 min and were then centrifuged at 800 x g for 20 min. The supernatant (bound fraction) was transfered to another set of new tubes and radioactivity determined (Ria Gamma 1271, LKB-Wallac, Finland) and concentrations calculated using spline curve

fitting of the data as described in Section 2.10.3.2.

Sensitivity (2 x standard deviation of the blank) was 31 pg and intraassay and interassay coefficients of variation were 6.7% and 9.1% respectively.

2.13.3. Pituitary hormones radioimmunoassays

2.13.3.1. Iodination of pituitary hormones

Pituitary hormones were iodinated using Chloramine-T method of Greenwood et al (1963).

(i) Preparation of Bio-Gel columns for gel filtration of iodinated hormone preparation

A. Preparation of columns

1. 10 ml disposable glass pipettes were used with the top 1 inch of the pipette cut-off.
2. Columns were thoroughly washed in Biochlor, rinsed and dried, siliconised with Cotasil, and washed in double distilled water and dried again.

B. Preparation of Bio-Gel

1. Approximately 50 g Bio-Gel was placed into 600 ml beaker and 300 ml PBS added and mixed with continued stirring for 2 - 3 hours.

2. The solution was allowed to settle and the excess buffer removed from above the gel. A new volume of PBS was added to the gel and stirring recommenced for 15 minutes.
3. Step 2 was repeated 3 times to wash the gel solution.

C. Building columns

1. A medium size glass bead was introduced into a 10 ml pipette which was placed in a column-holding device. A small piece of silastic tubing (4.7 mm O.D., 3.4 mm I.D.) was attached to the bottom of the pipette, and a screw clamp placed around the tube to control column flow.
2. Column was rinsed with PBS.
3. Column was filled with Bio-Gel by pouring the slurry of Bio-Gel into the column.
4. Allowed to settle (no movement of slurry apparent) to approximately 8 to 9 ml mark.
5. Released the clamp and allowed to settle further.
6. Column was kept wet at all times, and if too much Bio-Gel was in column, it was removed with a pipette to the recommended 1 ml mark.
7. Column was washed with 20 ml PBS.
8. 1.5 ml 5% albumin (Sigma) in PBS was gently pipetted onto column-bed, below buffer, for better recovery of iodinated protein.
9. Column was washed with another 20 ml PBS and capped with parafilm and stored at $4^{o}C$. Before use, column was allowed to warm to room temperature.
10. Columns were usually prepared 24 hours prior to use.

(ii) Reagents

1. Purified hormone:

   Highly purified hormone preparations of LH, FSH, TSH and prolactin were used. Details have been described separately in the procedures under each hormone.

2. Radioactive iodine:

   Sodium iodide (^{125}I) was obtained from Amersham, Australia. For each iodination 0.5 mCi in 12.5 ul was used.

3. Chloramine-T:

   A stock solution of Chloramine-T was prepared by dissolving 4 mg Chloramine-T in 5 ml PBS, immediately prior to each iodination.

4. Sodium metabisulphite ($Na_2S_2O_5$):

   Sodium metabisulphite (10 mg) was dissolved in 5 ml PBS prior to each iodination.

5. Transfer solution:

   50 mg of Potassium iodide was dissolved in 5 ml of 16% sucrose in PBS, prior to iodination.

6. Rinse solution:

   50 mg of Potassium iodide was dissolved in 5 ml of 8% sucrose in PBS, prior to iodination.

(iii) Iodination Procedures

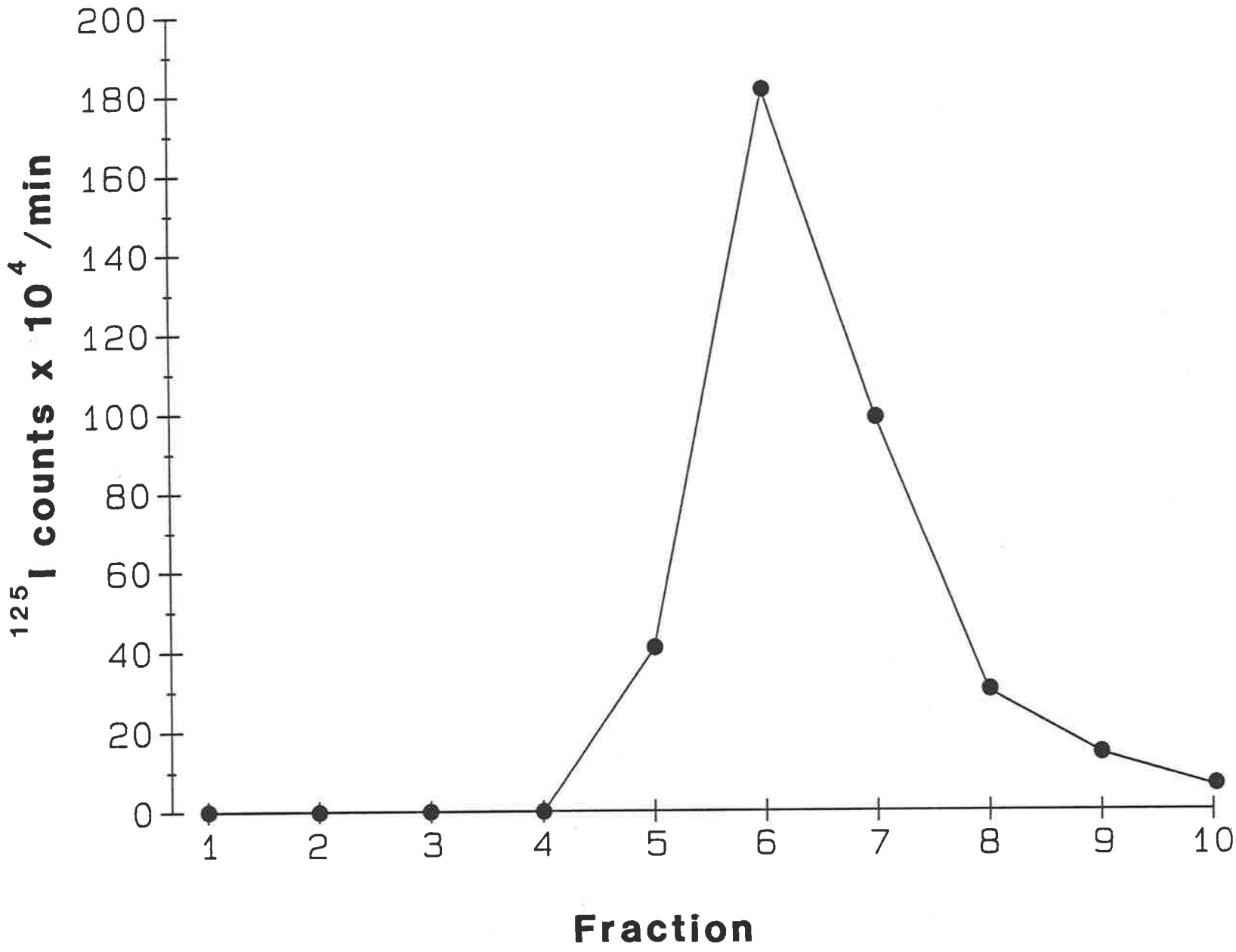
1. The amount of protein to be iodinated was dissolved in 25 ul PBS, in an Eppendorf tube. Quantities of specific proteins

used were 2.5 ug of LH, 5 ug of oFSH or 2.5 ug of rFSH, 2.5 ug of Prl and 2 ug of TSH.

2. 0.5 uCi ^{125}I Sodium iodide was added to the hormone and mixed well by tapping on the tube.
3. Chloramine-T stock solution (2 ug/5 ul) was added, mixed and left for 1 minute at room temperature.
4. Sodium metabisuphite (50ul) was added using TB syringe to stop the reaction.
5. After leaving for 1 minute the mixture was transfered with 100 ul of transfer solution, layered beneath the PBS on the surface of the Bio-Gel P-60 column and allowed to penetrate into the column. It was then rinsed with 100 ul of rinse solution and transfered on to the column.
6. The outlet of the column was opened and 0.5 ml samples were collected in tubes (12 x 75 mm) containing 100 ul 5% BSA solution (0.6 ml/tube). The volume was marked on each tube.
7. 10ul aliquot from each fraction was counted for radioactivity. The ^{125}I labelled hormone was eluted first, followed by the free ^{125}I. A typical iodination elution profile is shown in Figure 2.4.

The fractions with highest radioactivity in the hormone peak were retained and diluted in PBS to give approximately 20,000 CPM per 100 ul.

Figure 2.4. Levels of radioactivity in fractions eluted from a Biogel column used to separate bound and free ^{125}Iodine, after iodination of protein hormone using chloramine-T. The unbound free iodine was not eluted, and remained on the column. With such an elution profile, fractions 6 and 7 were used in radioimmunoassays.


200
180
160
140
120
100
80
60
40
20
0
^{125}I counts x 10^4/min
1
2
3
4
5
6
7
8
9
10
Fraction

2.13.3.2. Ovine Luteinizing hormone (oLH) radioimmunoassay

(i) General method

400 ul of plasma or standard hormone were placed in 12 x 75 mm glass vials using a reagent dispensor (Micromedic Systems, Horsham, PA, U.S.A.), along with 400 ul of antibody diluted in Gel PBS to give appropriate binding and displacement. Iodinated hormone (100 ul) was then added with another dispenser, and tubes shaken to ensure mixing of contents. Assay tubes were held at room temperature (22^{o}C) for 24 hours.

(ii) Separation of antibody and hormone from free hormone

Diluted serum (1:11 with PBS) containing second antibody (Antirabbit gamma globulins raised in sheep against rabbit gamma globulins) was then added to the tubes (100 ul) which were mixed well and held at room temperature for another 24 hours and then at 4^{o}C for a final 24 hour period. Anti-rabbit gamma globulins formed a precipitate with the antibody along with the bound fraction of the hormone. The free hormone was washed with 1.8 ml of PBS (at 4^{o}C) and decanted after centrifugation (2000 rpm for 20 min at 4^{o}C). The pellet containing the bound fraction in the precipitate was counted in the auto Riagamma counter.

(iii) Hormones

A highly purified form of Ovine LH (LER-1056-C2) used for

iodinations was a gift from Dr. L.E. Reichert Jr., Albany Medical College, Albany, New York, 12208, U.S.A.. Standards of 0, 0.0196, 0.039, 0.078, 0.1562, 0.3125, 0.625, 1.25, 2.5, 5, 10 ng/200 ul oLH, were prepared in Gel PBS, using ovine LH (NIADDK-oLH-24) obtained from NIAMDD, Bethesda, Maryland, U.S.A.

(iv) Antisera

The anti-ovine LH serum was obtained from Dr. D.R. Lindsay, University of Western Australia, Nedlands, Australia. The antiserum exhibited low cross-reactions with TSH (NIH-TSH-S8, 4.6%) prolactin (NIH.P.S12, 0.17%) FSH (NIH.FSH.S12, 0.92%) and growth hormone (NIH.GH.S11, 1.25%) (Oldham et al, 1979). The sensitivity of the assay using this antisera was 0.03 ng and the inter- and intra-assay coefficients of variation were 7.9% and 4.5% respectively.

(v) Calculation of results

A. Non-specific binding component

An estimate of the percentage of counts added which contribute to the bound fraction, in the absence of antibody, was required so that a correction could be applied to the calculation of actual counts bound. To assess this within each assay, 4 tubes containing 200 ul of Gel PBS 100 ul of ^{125}I LH and 400 ul of 0.75% normal rabbit serum in Gel PBS, were included.

B. Calculation of % bound

The percentage of total counts bound to antibody, including correction factor for NSB% was calculated as follows:

$$\% \text{ bound} = \frac{\text{Total counts bound to antibody}}{\text{Total counts added to the tube}} \times 100$$

Standard curves were constructed by plotting % bound against LH concentration. The % bound data was fitted in a spline curve plot of

$$\frac{B^{o} \text{ (\% bound in the absence of LH)}}{B^{x} \text{ (\% bound at each concentration of LH)}}$$

against LH concentration, from which test samples were quantitated. The counting and calculations were carried out in an LKB-Wallac automatic microcomputer-controlled two channel gamma counter dedicated to RIA applications.

2.13.3.3. Rat Luteinizing hormone (rLH) radioimmunoassay

General method, separation of antibody bound from free hormone and calculation of the results are similar to that used for oLH (Section 2.13.3.2.) except the volume of plasma used in the assay was 200 ul.

Materials for the rLH radioimmunoassay were obtained from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK), USA. Immuno-reagents were elaborated by Dr A.F. Parlow, Director, Pituitary Hormones and Antisera Centre, Harbour-UCLA Medical Centre, Torrance, California, USA and are described in Technical Report No. 143 of the Centre.

A. Hormone

Highly purified rat Luteinizing hormone antigen (NIADDK-rLH-I-6) was used for iodinations. Standards of 0, 0.07, 0.15, 0.31, 0.62, 1.25, 2.50, 5.00, 10.00 ng/200 ul rLH, were prepared in Gel PBS, using rat LH (NIADDK-rLH-RP-2).

B. Antiserum

The anti-rat LH serum raised in rabbits was NIADDK-anti-rLH-S-7. The antiserum exhibited low cross-reactions with TSH (NIADDK-rTSH-I, 5.2%), prolactin (NIADDK-rPRL-I, 0.01%), FSH (NIADDK-rFSH-I, 0.15%) and growth hormone (NIADDK-rGH-I, 0.004%) (Technical Report No. 143, as detailed above). The sensitivity of the assay using this antisera was 0.3 ng and the inter- and intra-assay coefficients of variation were 7.4 % and 5.0 % respectively.

2.13.3.4. Ovine Follicle Stimulating hormone (oFSH) radioimmunoassay

General method, separation of antibody bound from free hormone and calculation of the results are similar to that used for oLH

(Section 2.13.3.2.) except the volume of plasma used in the assay was 200 ul.

A. Hormone

Standard solutions of FSH were prepared from purified ovine (NIAMDD-oFSH-15) FSH obtained from NIAMDD, Bethesda, MD, U.S.A.. Standards of 0, 0.7812, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100 ng FSH/200 ul were prepared in Gel PBS. A highly purified form of Ovine FSH (LER-1976-A2) used for iodination was a gift from Dr. L.E. Reichert Jr.

B. Antiserum

The antiserum was obtained from Dr. S.S. Lynch, Birmingham and Midland Hospital for Women, Birmingham, which was raised in rabbits against hFSH. The cross reactivity of the antisera with oFSH was 100% whereas with rFSH it was 76%. Ovine LH, TSH, GH, prolactin, rat LH, TSH, GH and prolactin showed less than 0.2% crossreactivity (McNeilly et al, 1976). The sensitivity of the assay using this antisera was 0.5 ng and inter and intra assay coefficients of variation were 8.9% and 6.0% respectively.

2.13.3.5. Rat Follicle Stimulating hormone (rFSH) radioimmunoassay

General method, separation of antibody bound from free hormone and calculation of the results are similar to that used for oLH (Section 2.10.3.2.) except the volume of plasma used in the assay was

200 ul.

Materials for the rFSH radioimmunoassay were obtained from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK), USA. Immuno-reagents were elaborated by Dr A.F. Parlow, Director, Pituitary Hormones and Antisera Centre, Harbour-UCLA Medical Centre, Torrance, California, USA and are described in Technical Report No. 144 of the Centre.

A. Hormone

Highly purified rat FSH antigen (NIADDK-rFSH-I-6) was used for iodinations. Standards of 0, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25.00, 50.00 ng/200 ul rLH, were prepared in Gel PBS, using rat FSH (NIADDK-rFSH-RP-2).

B. Antiserum

The anti-rat FSH serum raised in rabbits was NIADDK-anti-rFSH-S-11. The antiserum exhibited low cross-reactions with TSH (NIADDK-rTSH-I, 1.67%), prolactin (NIADDK-rPRL-RP-2, 0.06%), LH (NIADDK-rLH-I-5, 0.01%) and growth hormone (NIADDK-rGH-I-4, 0.02%) (Technical Report No. 144, as detailed above). The sensitivity of the assay using this antisera was 0.5 ng and the inter- and intra-assay coefficients of variation were 8.2 % and 5.6 % respectively.

2.13.3.6. Rat Prolactin (rPRL) radioimmunoassay

General method, separation of antibody bound from free hormone and calculation of the results are similar to that used for oLH (Section 2.13.3.2.), except the volume of plasma used was 50 ul.

Materials for the rPRL radioimmunoassay were obtained from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK), USA. Immuno-reagents were elaborated by Dr A.F. Parlow, Director, Pituitary Hormones and Antisera Centre, Harbour-UCLA Medical Centre, Torrance, California, USA and are described in Technical Report No. 120 of the Centre.

A. Hormone

Highly purified rat PRL antigen (NIADDK-rPRL-I-5) was used for iodinations. Standards of 0, 0.098, 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00 ng/50 ul rPRL, were prepared in Gel PBS, using rat PRL (NIADDK-rPRL-RP-3).

B. Antiserum

The anti-rat PRL serum raised in rabbits was NIADDK-anti-rPRL-S-9. The antiserum exhibited less than 0.01 % cross-reactivity with TSH (NIADDK-rTSH-I-3), LH (NIADDK-rLH-I-4), FSH (NIADDK-rFSH-I-3) and growth hormone (NIADDK-rGH-I-3) (Technical Report No. 120, as detailed above). The sensitivity of the assay using this antisera was 0.8 ng and the inter- and intra-assay

coefficients of variation were 8.5 % and 4.3 % respectively.

2.13.3.7. Ovine Thyroid Stimulating hormone (oTSH) radioimmunoassay

General method, separation of antibody bound from free hormone and calculation of the results are similar to that used for oLH (Section 2.13.3.2.), except the volume of plasma used was 100 ul.

Materials for the radioimmunoassay were obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), USA. Immuno-reagents were elaborated by Dr A.F. Parlow, Director, Pituitary Hormones and Antisera Centre, Harbour-UCLA Medical Centre, Torrance, California, USA and are described in Technical Report No. 139 of the centre.

A. Hormone

Highly purified bovine TSH antigen (NIADDK-bTSH-I-1) was used for iodinations. Standards of 0, 0.39, 0.78, 1.56, 3.12, 6.25, 12.50, 25.00, 50.00, 100.00 ng/100 ul TSH, were prepared in Gel PBS, using bovine TSH (NIADDK-bTSH-I-1).

B. Antiserum

The anti-ovine TSH serum raised in rabbits was NIADDK-anti-oTSH-1. The antiserum exhibited low cross-reactions with LH (NIAMDD-oLH-23, 0.18%), FSH (oFSH, AFP-5679-C, 0.03%), prolactin (oPRL, AFP-4328-C, 0.02%) and growth hormone (oGH, AFP-5285-C, 0.07%).

2.14. HISTOLOGY

2.14.1. Fixatives

Testicular tissue was preserved in Bouins solution and Kidney tissue was preserved in Buffered Formalin fixative.

Bouins Solution

75 ml saturated Picric Acid

(1.5 gram percent in distilled water at 30 ^{o}C)

25 ml Formalin

5 ml Glacial Acetic Acid

Tissue was placed in Bouins solution for 24 hours and then transferred to 70% Ethanol for storage.

Buffered Formalin

8.0 g $NaH_2PO_4.2H_2O2$

13.0 g Na_2HPO_4

200 ml Formalin

1800 ml Distilled Water

Tissue was placed into Buffered Formalin, and remained in the fixative during storage.

2.14.2. Tissue Processing

Blocks of tissue were placed in Tissue-Tek cassettes and dehydrated and cleared in an automatic processor in preparation for embedding in paraffin wax as follows:

1. 70% Ethanol	1 hour
2. 80% "	1 hour
3. 95% " I	1/2 hour
4. 95% " II	1 hour
5. Abs " I	2 hours
6. Abs " II	2 hours
7. 1:1 Abs Ethanol/ Chloroform	1 hour
8. Chloroform I	2 hours
9. Chloroform II	2 hours
10. Wax I	2 hours
11. Wax II	2 hours

Tissue sections of 7 um thick were cut with a microtome and stained with haematoxylin and eosin as follows:

1. Xylol	2 minutes
2. Xylol	2 minutes
3. Absolute alcohol	1 minute
4. 80% alcohol	1 minute
5. 30% alcohol	1 minute
6. Running tap water	1 minute
7. Mayers haematoxylin	1.5 minutes
8. Running tap water	1 minute
9. 1% acid alcohol	few drops till pink
10. Running tap water	10 minutes
11. Eosin	7 seconds

12.	80% alcohol	rinse
13.	Absolute alcohol	2 minutes
14.	Absolute alcohol	2 minutes
15.	Xylene	2 minutes
16.	Xylene	2 minutes
17.	Mount in DPX	

2.15. IMMUNOHISTOCHEMISTRY

2.15.1. Fixatives

Tissue to be examined immunohistochemically was placed in 2% Formaldehyde in 0.1 M sodium phosphate buffer pH 7.0, containing 15% picric acid as described by Stefanini et al (1967) :

Picric Acid/ Formaldehyde Fixative

50 ml AR Formaldehyde (40% solution)

500 ml 0.2 M sodium phosphate buffer, pH 7.0

150 ml saturated aqueous solution of picric acid filtered through Whatman No. 1 filter paper

Made up to 1 litre with distilled water, and stored at 4 ^{o}C.

Sodium Phosphate buffer

Stock solution A : $NaH_2PO_4.2H_2O$ 31.202 g/l

Stock solution B : Na_2HPO_4 28.392 g/l

For pH 7.0, 39 ml of solution A and 61 ml of solution B were

mixed and adjusted to pH with 10 M NaOH or HCl.

Fixation :

Pieces of tissue were trimmed so that at least one dimension was 5mm or less, and placed into Picric Acid Formaldehyde which had been maintained at ice temperature (< 4 ^{o}C). Tissue was kept in this fixative for 16-24 hours.

Dehydration, Clearing and Rehydration

Tissue was placed in 80% Ethanol, with changes every 15 minutes until the yellow colouration of the picric acid was removed (4-5 changes). The tissue was then dehydrated, cleared and rehydrated as follows :

1. 95% Ethanol	30 minutes
2. 100% " I	"
3. 100% " II	"
4. Xylene I	"
5. Xylene II	"
6. 100% Ethanol I	"
7. 100% " II	"
8. 100% " III	"
9. 95% "	15 minutes
10. 80% "	"
11. 50% "	"
12. 25% "	"
13. Distilled Water	"
14. Phosphate Buffered Saline	"

Tissue was stored in 30% sucrose, 0.1% sodium azide in

phosphate buffered saline.

PBS/Sucrose/Azide

8.5 g	NaCl
1.07 g	Na_2HPO_4
0.39 g	$NaH_2PO_4.2H_2O$
300.00 g	Sucrose
1.00 g	Na Azide

Made up to 1 litre with distilled water.

2.15.2. Tissue Processing

Cryostat sections were cut at 5 um , dessicated under vacuum for 30 minutes, and stored sealed at 4 ^{o}C for up to 1 week if necessary. Tissue was then processed as follows :

1. Pre-incubated for 30 minutes with normal sheep serum (10% in PBS)
2. Excess sheep serum drained off slides
3. Antisera added to appropriate slides :

   Rabbit-anti-oFSH (see 2.10.3.4.)

   50 ul/slide at 1:400 dilution

   Rabbit-anti-oLH (see 2.10.3.2.)

   50 ul/slide at 1:400 dilution

   Rabbit-anti-oPRL (NIAMDD-anti-oPRL-1)

   30 ul/slide at 1:400 dilution

   Mouse-anti-h B-Endorphin (see Gramsch et al, 1983; gift to Dr J. Furness per A. Hertz)

   50 ul/slide at 1:400 dilution

4. Incubated overnight at room temperature in humidity boxes
5. Gently washed in PBS with stirring for 15 minutes
6. Fluorescent conjugated second antibodies added to appropriate slides :

   Sheep-anti Rabbit-FITC (Wellcome Diagnostics)

   40 ul/slide at 1:80 dilution

   Goat-anti Mouse-FITC (Cappel)

   40 ul/slide at 1:80 dilution
7. Incubated for 1 hour at room temperature in humidity boxes
8. Excess second antibodies washed off with PBS.
9. Gently washed in PBS with stirring for 15 minutes
10. Mounted in Buffered Glycerol (pH 8.6)

Buffered Glycerol (pH 8.6)

Solution A - 0.5 M sodium carbonate

53 g/l Na_2CO_3 (pH 11.5)

Solution B - 0.5 M sodium bicarbonate

42 g/l $NaHCO_3$ (pH 8.3)

Solution C - Glycerol

One part solution B placed into a beaker and adjusted to pH 8.6 with solution A. Two parts glycerol added to buffer solution.

2.16. STATISTICAL ANALYSIS

All data is presented as mean ± standard error of the mean (SEM), unless otherwise stated. The probability of significant differences between groups was calculated by Student's t-test. Treatment effects were analysed by Analysis of Variance (ANOVA) or Analysis of Covariance (ANCOVA). Differences in tissue graft survival were tested for significance with X^2-tests as described by Finney (1948) and Latscha (1953).

CHAPTER 3: THE RODENT TESTIS AS AN IMMUNOLOGICALLY PRIVILEGED SITE

3.1. INTRODUCTION

Previous studies on the immune privileged status of the rodent testis have highlighted the likely importance of some factor or factors in the interstitial region of the testis. The experiments to be reported in this chapter were undertaken to initially establish a routine transplantation protocol, to confirm the immune privileged status of the rodent testis, and to then examine components of the interstitial region of the rodent testis that might be important in creating the proposed immune privileged status of this site.

3.2. CHOICE OF TISSUE GRAFTS

The potential use of endocrine tissue grafts in an immunologically privileged testis to treat hormonal disorders or to stimulate endocrine systems requires that such grafts should continue to secrete hormones after transplantation and to respond to normal endocrine stimuli. Two tissues were chosen for use in these studies which would allow various endocrine parameters to be monitored. Initially the pituitary gland, which is involved in numerous endocrine activities, was chosen because it secretes some hormones when specific stimuli are provided (eg. LH, FSH), and others when specific inhibitors are removed (eg. Prolactin). In later experiments, the thyroid gland was chosen for investigation because it not only secretes hormones, but also concentrates iodine from the blood, which can be readly monitored with the administration of radioactive iodine.

3.3. PITUITARY TRANSPLANTS

Three methods of pituitary preparation were tested. Initially, isolated pituitaries were treated with collagenase digestion to procure isolated cells which have been shown to retain endocrinological activity in vitro (Vale et al, 1972). In a second experiment, similar cells were obtained using trypsin digestion in place of collagenase to investigate the possibility that inappropriate enzyme treatment might affect cell function after transplantation. In a third experiment, enzyme digestion was avoided altogether,with the use of quarters of whole pituitaries.

Donor pituitaries were obtained for transplantation from castrated male rats using the method detailed in 2.3.2. These animals were used because the lack of steroid feedback on the pituitary should have rendered the pituitaries hyperactive in terms of gonadotrophin secretion. In the first two experiments, recipients were hypophysectomised at the time of transplantation. However, this in itself may affect graft survival and thus, in the third experiment, both intact and hypophysectomised recipients were used. At this time, Selawry and Whittington (1984) published a report suggesting that intratesticular grafts of pancreatic islets were more successful in cryptorchid than in scrotal testes, and thus this third experiment also included animals with cryptorchid testes as recipients.

3.3.1. Experimental Procedures

3.3.1.1. Animals

Male Porton rats were maintained in the departmental facilities under a constant 12 hours light : 12 hours dark. A standard pelleted diet (Milling Industries, Adelaide) and water were available ad libitum.

3.3.1.2. Experiment 1. Collagenase-dispersed cells

Pituitaries were removed from twenty male donors (200-250 grams) sacrificed under ether anaesthesia, and individual cells isolated using collagenase dispersion as described in 2.4.2. This digestion yielded 10.5 X 10^6 cells (determined with a Neubauer haemocytometer) which were suspended in 2 ml Dulbecco's PBS. Twenty male recipients (140-290 grams), hypophysectomised one week before transplantation as detailed in 2.3.2., were randomly allocated into four groups of five animals. One group was not transplanted and served as a control group, while the other three groups were transplanted with 50, 100 and 200 ul respectively of the cell suspension (250 000, 500 000, and 1 000 000 cells) into one testis as described in 2.3.7.2. All animals were weighed weekly after transplantation and at three weeks after transplantation, surviving animals were re-weighed and venous blood samples were obtained from both testes and the posterior vena cava as described in 2.3.5. Prolactin levels were measured in these samples by radioimmunoassay as described in 2.13.3.6.

3.3.1.3. Experiment 2. Trypsin-dispersed cells

Pituitaries were removed from twenty male donors (230-300 grams) as in experiment 1 above. Individual cells were isolated using trypsin digestion as described in 2.4.3. This digestion yielded 20 X 10^6 cells in 4 ml Dulbecco's MEM. Twelve male recipients (260-320 grams) were hypophysectomised at the time of transplantation as detailed in 2.3.2. All animals were transplanted with 200 ul of the cell suspension (1 000 000 cells) into one testis as described in 2.3.7.2. At this time cells were also placed into culture as described in 2.4.4.1. To assess their viability at the time of transplantation, these cells were stimulated with LHRH as described in 2.4.4.2. Three weeks after transplantation, surviving animals were reweighed and venous blood samples were obtained and Prolactin levels monitored as for experiment 1 above.

3.3.1.4. Experiment 3. Quarters of whole pituitaries

Ten pituitaries were obtained from adult male donors (270-300 grams) as described above, and the anterior pituitary cut into quarters. Thirty adult male recipients (230-330 grams) were randomly divided into six groups. Three of these groups were hypophysectomised at the time of transplantation as described in 2.3.2., and three were left intact. With each of these two treatments, one group received intramuscular transplants of one pituitary quarter into the hind leg, one group received intratesticular transplants of a pituitary quarter into one testis in the scrotum, and the third group received transplants of a pituitary quarter into one testis made cryptorchid as

described in 2.3.3. Transplants were performed as described in 2.3.7.2. Three weeks after transplantation, surviving animals were reweighed and blood samples obtained as in experiments 1 and 2 above. Animals were then castrated and further blood samples taken after another three weeks. Prolactin, LH and FSH levels were measured using radioimmunoassays as described in 2.13.3.6., 2.13.3.3. and 2.13.3.5. respectively.

3.3.2. Results

3.3.2.1. Experiment 1. Collagenase-dispersed cells

There was considerable mortality amongst the animals in this experiment. Only one animal in the control group of hypophysectomised, non-transplanted rats survived the three week period, and this animal lost 20 grams in body weight during this period of time. All other animals in this group rapidly lost a similar amount of weight in a considerably shorter time period and died in weeks 1 and 2 of the experiment. None of the animals receiving the lowest transplantation dosage of 250,000 cells survived past the first week. Two out of the five animals receiving the 500,000 cell transplants died in week 1, as did three of the five animals receiving the 1,000,000 cell transplants. The surviving animals in these two groups either sustained their initial weight or marginally gained or slightly lost weight over the three week period of study (Table 3.1.). No difference in prolactin levels could be detected in any of the samples obtained from these animals as seen in Table 3.1.

Table 3.1. Changes in body weight of individual animals over three weeks after tranpslantation of collagenase-dispersed pituitary cells into the testis of hypophysectomised male rats, and plasma prolactin levels at three weeks after transplantation.

Transplant	Change in Body Weight (g)	Plasma Prolactin (ng/ml) Grafted Testis	Non-grafted Testis	Peripheral
500,000 cells	+12	14	15	16
	+11	11	15	10
	- 6	7	-	5
1,000,000 cells	- 2	10	11	11
	+ 1	-	10	6

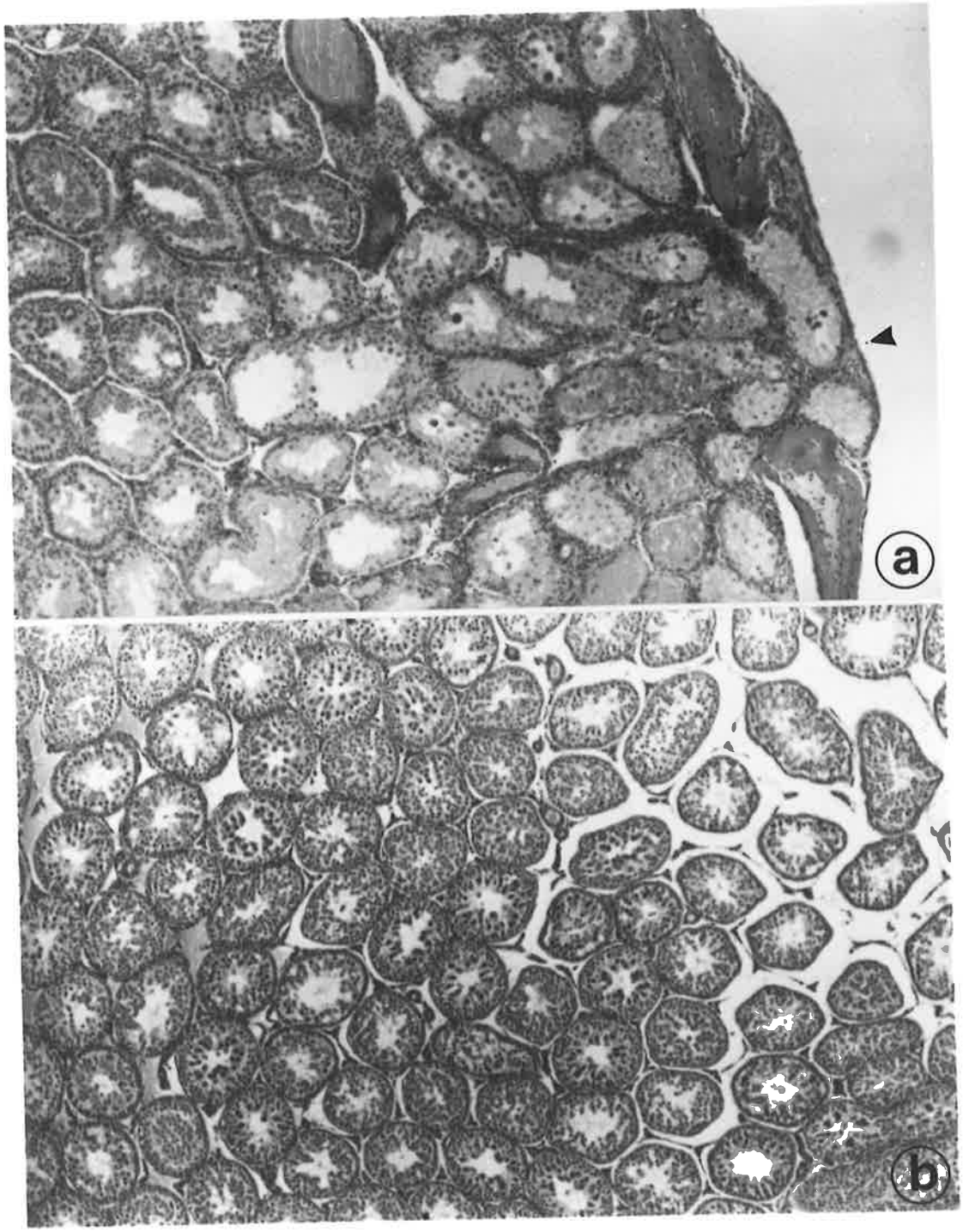
- : no sample obtained

Representative sections from the testis of an animal receiving 1,000,000 cells are shown in Figure 3.1. The graft site in the testis is clearly marked by the small group of tubules that have pushed out through the entry hole made in the tunica albuginea (see Figure 3.1.a). However, no grafted cells can be seen in the region of transplantation, and tubules in this region and more generally in the testis (Figure 3.1.b) are typical of tubules seen in untreated hypophysectomised animals. There is no evidence for the actions of any gonadotrophins in these sections (from local or systemic sources), suggesting that grafts have not been technically successful. While there is no evidence of any immune reaction, there is also no evidence of any graft tissue.

3.3.2.2. Experiment 2. Trypsin-dispersed cells

Trypsin-dispersed cells in culture did respond to the addition of

Figure 3.1. Representative sections from the testis of a hypophysectomised rat three weeks after intratesticular transplantation of 1,000,000 collagenase-isolated pituitary cells. (a) The graft site is clearly marked by a small group of tubules that have pushed out through the entry hole in the tunica albuginea (▶). However, no graft cells are visible in the region of transplantation. (b) Tubules are typical of those seen in the testes of hypophysectomised animals and there is no evidence for the action of any graft-derived gonadotrophins. X 80.


a
b

LHRH to the culture medium as seen in Table 3.2. suggesting that cells were viable at the time of transplantation. In this experiment, eight of the twelve animals survived the three weeks after transplantation, although all of these animals lost weight in this period (Table 3.3.). Once again, there was no clear evidence that concentrations of prolactin in the veins draining the testis containing the transplanted cells were any higher than in the venous blood from the ungrafted testis or from peripheral blood (Table 3.3.).

Table 3.2. The secretion of LH by cultures of trypsin-isolated rat pituitary cells under basal and LHRH-stimulated conditions. Values are given as Mean ± SEM (n=4).

Treatment	LH (ng/ml)
Control	0.11 ± 0.04
+ LHRH	2.93 ± 0.42[a]

a: significantly different from Control values ($P < 0.05$, students t-test).

Table 3.3. Changes in body weight over three weeks after tranpslantation of trypsin-dispersed pituitary cells into the testis of hypophysectomised male rats, and plasma prolactin levels at three weeks after transplantation. Values are given as Mean ± SEM (n=8).

Change in Body Weight (g)	Plasma Prolactin (ng/ml) Grafted Testis	Non-grafted Testis	Peripheral
- 43 ± 12	11 ± 6	12 ± 4	11 ± 5

The histological examination did not locate any of the grafted

cells, and as in the testes of animals transplanted with collagenase-isolated cells, there was no evidence for any immunological reaction. Testes were very similar in appearance to those shown in Figure 3.1.a and 3.1.b.

3.3.2.3. Experiment 3. Quarters of whole pituitaries

As in earlier experiments, considerable mortality of hypophysectomised animals affected the outcome of this experiment. While all normal animals survived and continued to gain weight (Figure 3.2.) during the period of study, only 4 of the 15 hypophysectomised animals survived (one intramuscular, two scrotal intratesticular and one cryptorchid intratesticular graft recipients). These animals lost weight during the three weeks of study (Figure 3.2). There was no difference in body weight gain between the different groups of normal rats, and no difference in body weight loss of the hypophysectomised rats, irrespective of the location of the tissue graft.

Levels of LH (Figure 3.3.), FSH (Figure 3.4.) and Prl (Figure 3.5.) were significantly reduced by hypophysectomy. Transplantation did not affect the levels of any of these hormones in either normal or hypophysectomised animals. Following castration, levels of LH and FSH rose significantly in normal animals. Prolactin levels were significantly reduced in normal animals following castration.

Whole tissue grafts are much more readily identified than grafts of isolated cells, and pituitary grafts were found in some of the graft recipients in this experiment. No grafts were recovered from intramuscular sites, or from cryptorchid testes, although recovery

Figure 3.2. Percentage change in body weight of normal (solid bars) and hypophysectomised (hatched bars) rats over the three week period after transplantation of one quarter of a rat pituitary into a leg muscle, a scrotal testis, or a cryptorchid testis. Numbers of animals surviving in each group are indicated in brackets.

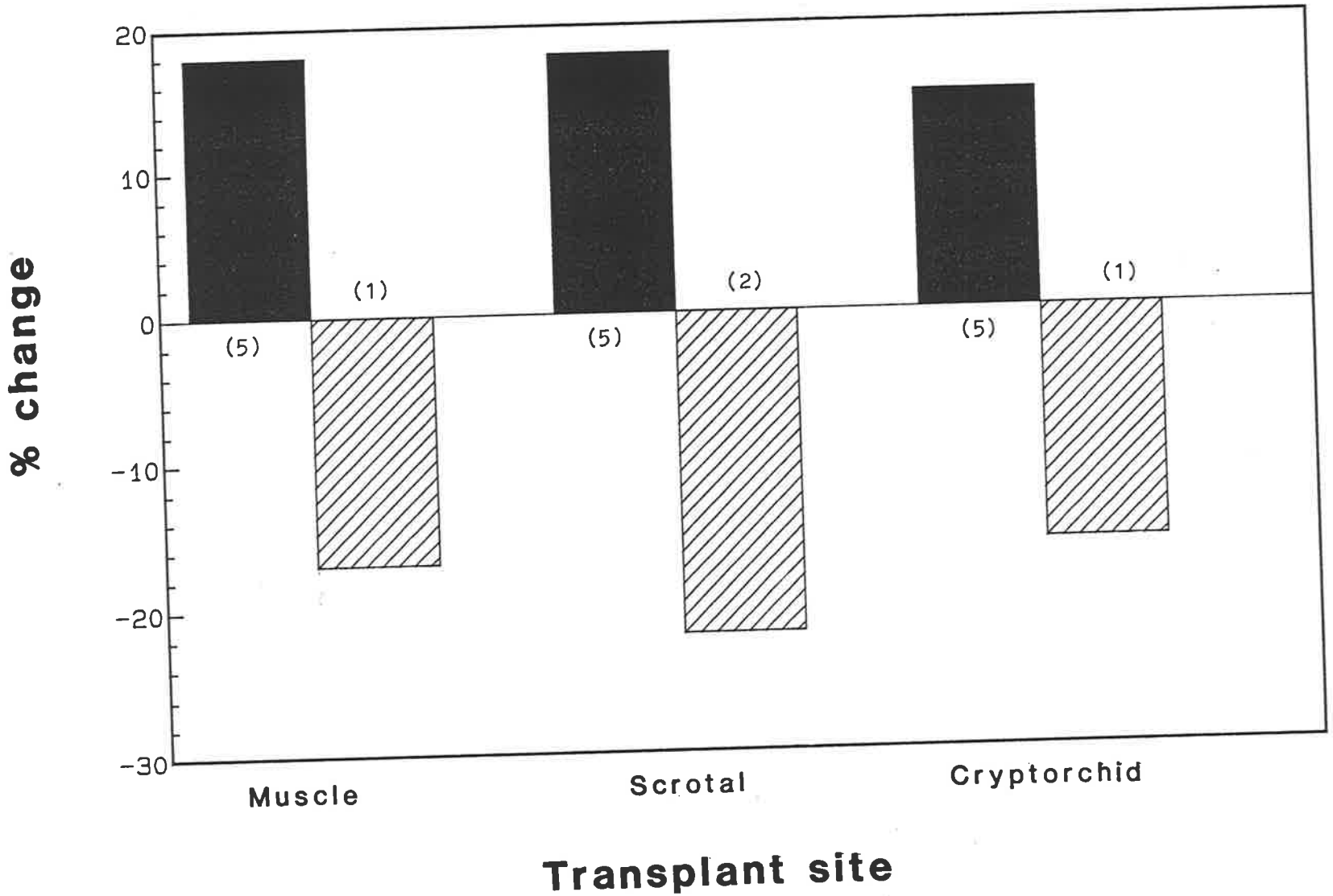

20
10
0
-10
-20
-30
% change
(5)
(1)
(5)
(2)
(5)
(1)
Muscle
Scrotal
Cryptorchid
Transplant site

Figure 3.3. Percentage change in plasma LH levels in normal (solid and open bars) and hypophysectomised (hatched and cross-hatched bars) rats, following transplantation of one quarter of a rat pituitary into a leg muscle, a scrotal testis, or a cryptorchid testis : (a) during the three weeks between transplantation and castration (solid and hatched bars); and (b) over the three week period after castration (open and cross-hatched bars).

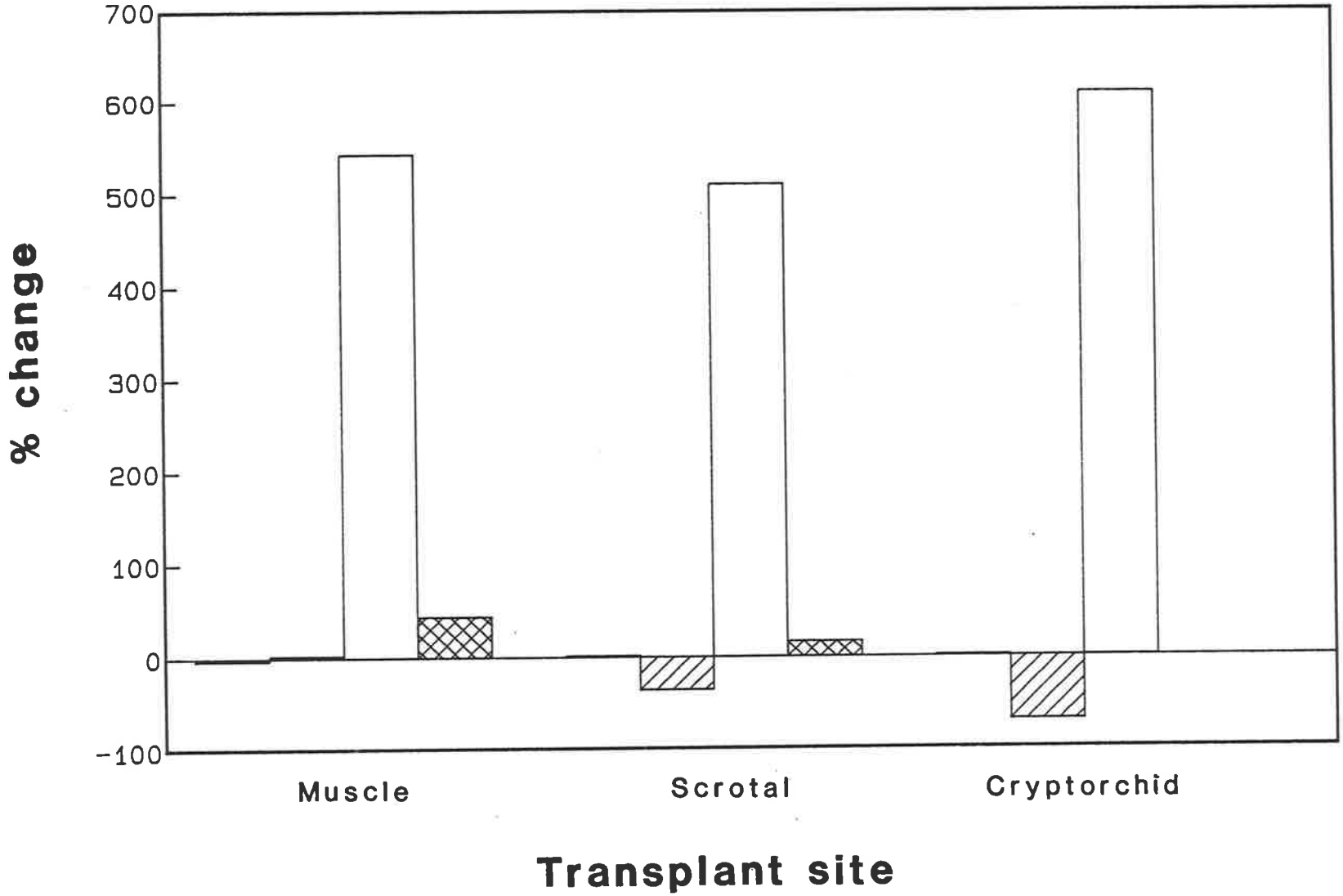

700
600
500
400
300
200
100
0
-100
% change
Muscle
Scrotal
Cryptorchid
Transplant site

Figure 3.4. Percentage change in plasma FSH levels in normal (solid and open bars) and hypophysectomised (hatched and cross-hatched bars) rats, following transplantation of one quarter of a rat pituitary into a leg muscle, a scrotal testis, or a cryptorchid testis : (a) during the three weeks between transplantation and castration (solid and hatched bars); and (b) over the three week period after castration (open and cross-hatched bars).

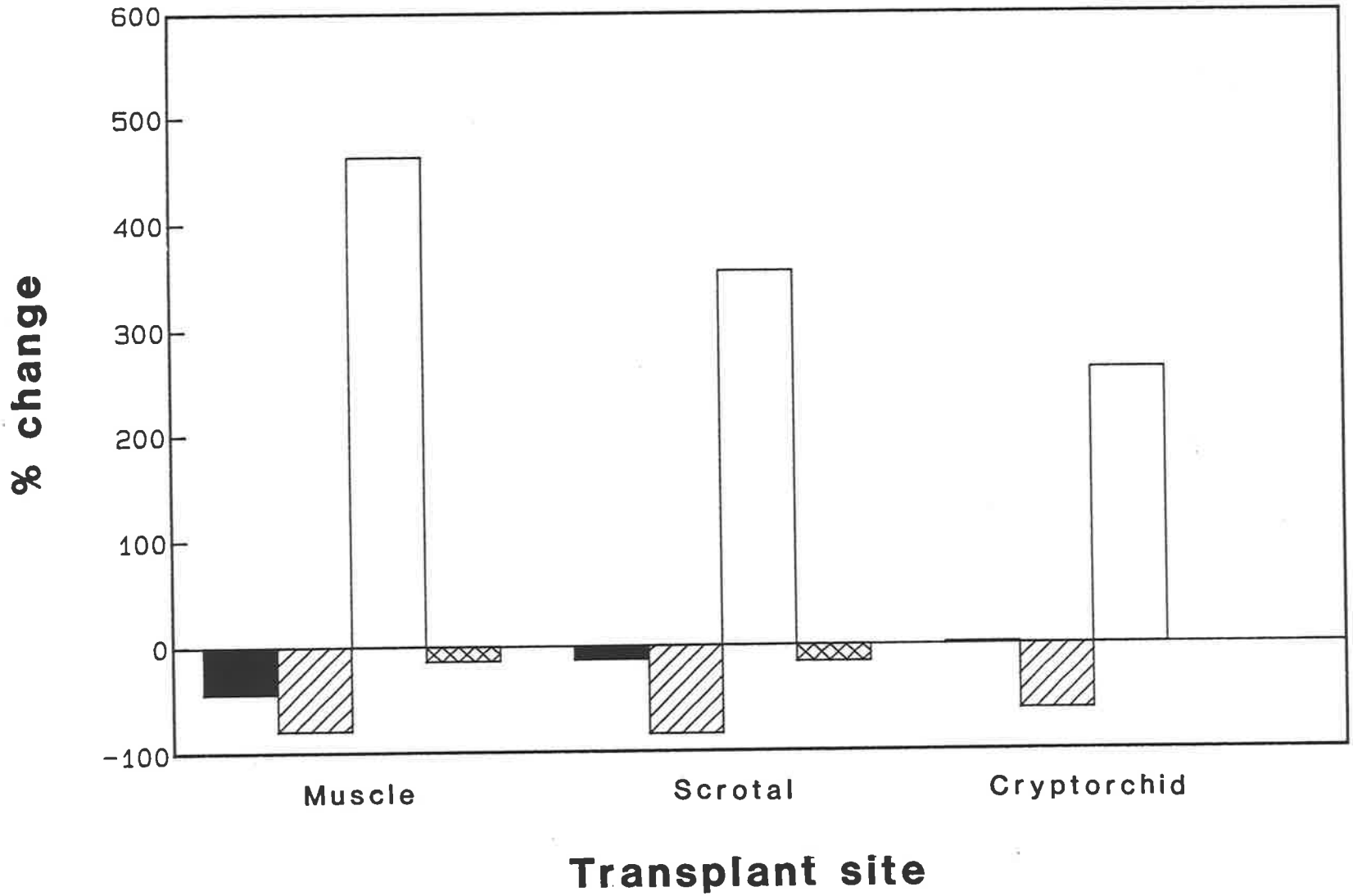
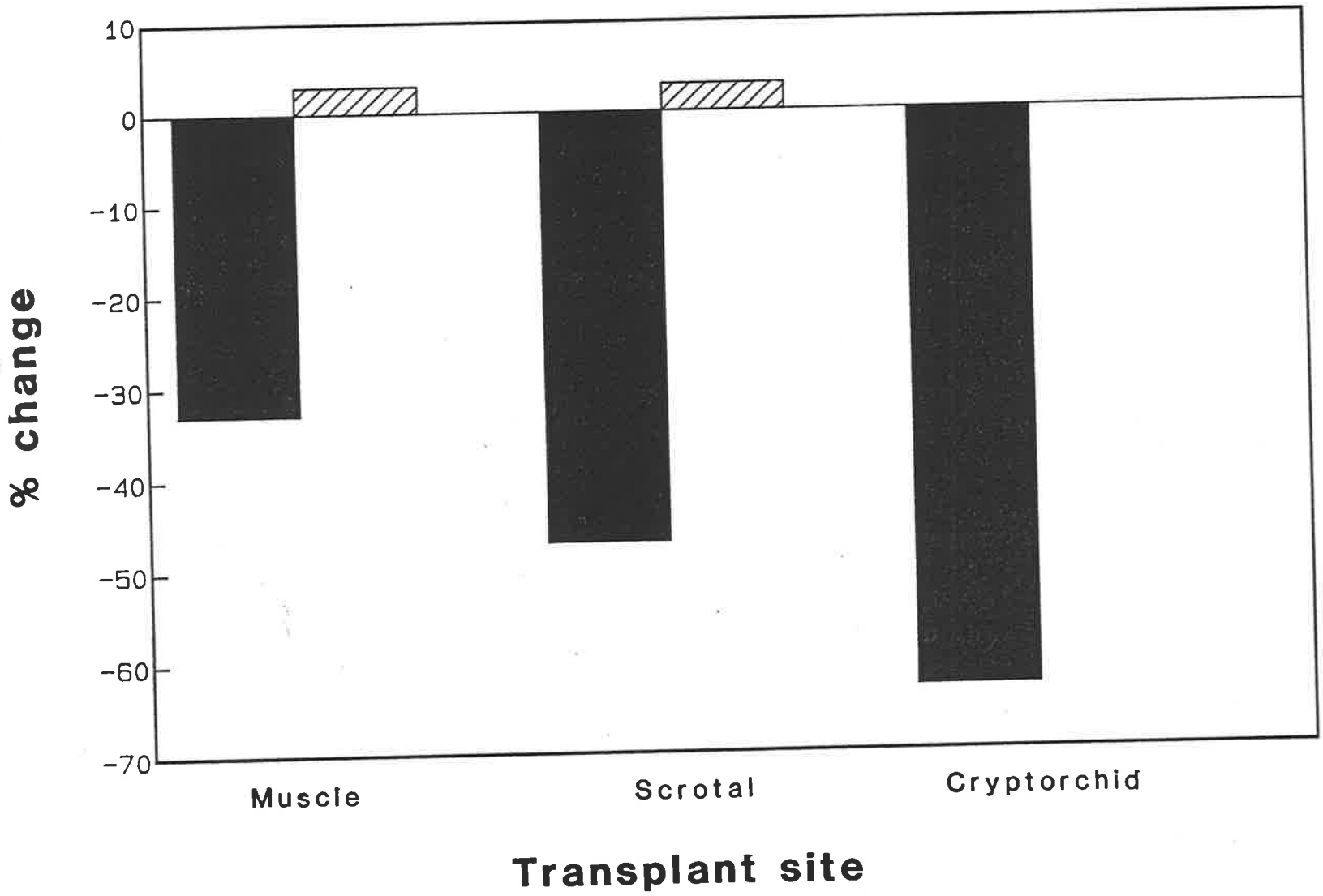

600
500
400
300
200
100
0
-100
% change
Muscle
Scrotal
Cryptorchid
Transplant site

Figure 3.5. Percentage change in plasma Prolactin levels in normal (solid bars) and hypophysectomised (hatched bars) rats over the 3 week period after castration and removal of intratesticular pituitary grafts.

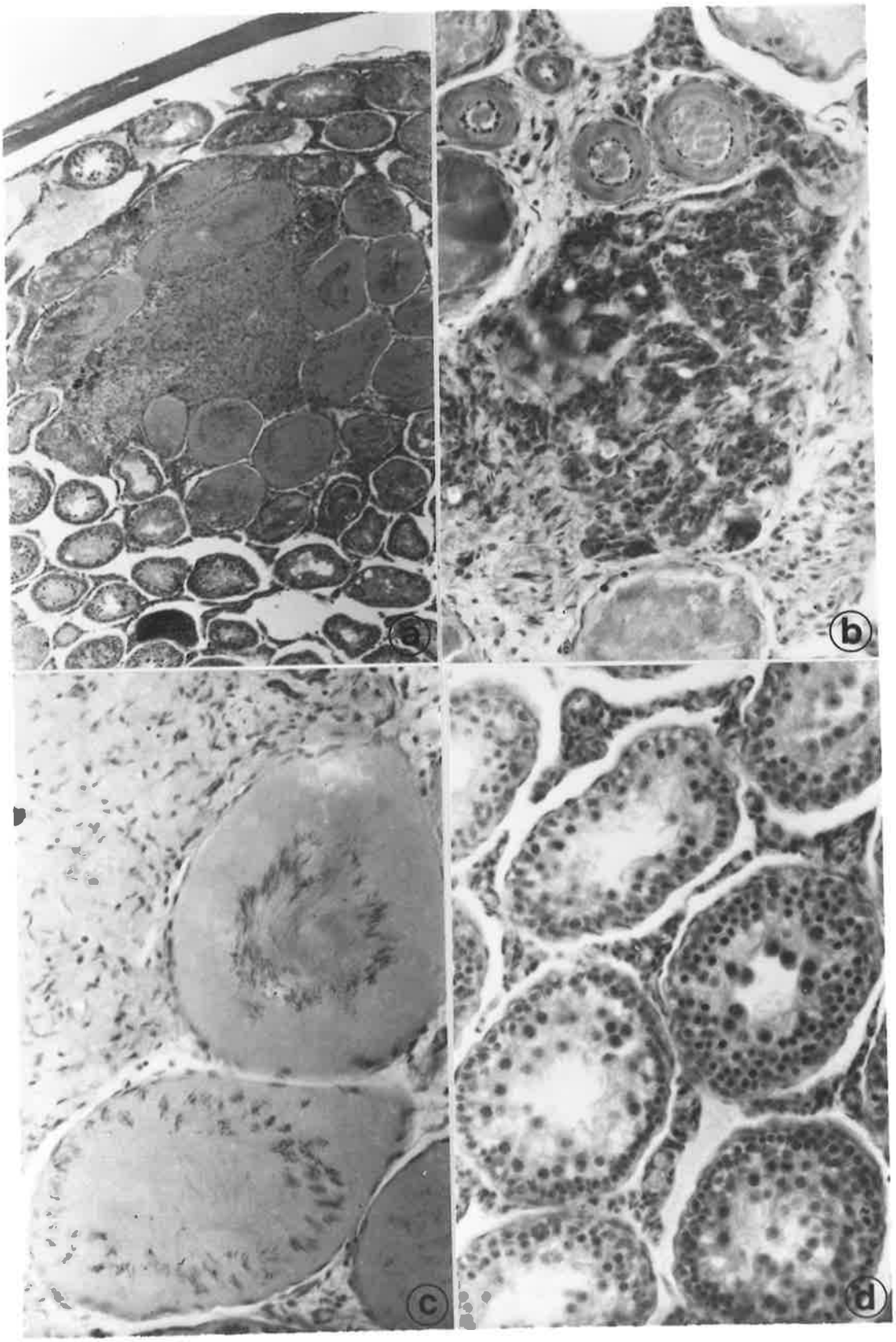

10
0
-10
-20
-30
-40
-50
-60
-70
% change
Muscle
Scrotal
Cryptorchid
Transplant site

from these testes was hampered by technical problems. However, grafts were found in animals transplanted with pituitary grafts into scrotal testes, particularly in hypophysectomised animals. Figure 3.6. shows sections from hypophysectomised recipients, and as seen in Figure 3.6.a, the pituitary tissue is readily identified. While much of the central region of the graft appears necrotic, peripheral regions are quite healthy, and Figure 3.6.b shows a region of typical pituitary tissue on the edge of a graft. Of particular interest is the ring of tubules around the graft (Figure 3.6.a) which are shown in greater detail in Figure 3.6.c. These tubules do not appear to contain any Sertoli cells or developing germ cells, and there is no identifiable lumen in most of them. However, the central region contains numerous sperm and both sperm heads and tails are clearly seen. This is unlikely to be a fixation artifact, since tubules in the rest of the testis (Figures 3.6.a and 3.6.d) are typical of those found in the testes of hypophysectomised animals, and do not show any of these peculiar characteristics.

3.3.3. Discussion

The results of these experiments were disappointing to say the least. That transplanted-hypophysectomised animals in Experiment 1 were able to maintain body weight while non-grafted hypophysectomised animals lost weight was encouraging. However, the lack of any evidence for prolactin secretion by such grafts was discouraging. There is evidence that inappropriate enzyme treatment during isolation of cells can result in a decrease in hormone secretion and more importantly in receptors to trophic hormones(Vale et al, 1972; Smith and Vale, 1980).

Figure 3.6. Histological sections from the testes of hypophysectomised rats three weeks after intratesticular transplantation of a quarter of a rat anterior pituitary. (a) The tissue graft is readily identified below the tunica albuginea, and is surrounded by seminiferous tubules. X 80. (b) Healthy pituitary tissue is seen in the peripheral regions of the graft. X 320. (c) Tubules in close proximity to the graft do not appear to contain any lumen, germinal elements or Sertoli cells, and yet sperm are clearly seen. X 320. This is unlikely to result from a fixation artifact since tubules in other regions of the same testis (d) are typical of those found in the testes of hypophysectomised animals and do not show the same characteristics. X 320.


a
b
c
d

Experiment 2 was designed to address this problem. While collagenase treatment may have affected the viability of tissue grafts in experiment 1, there was no suggestion from experiment 2 that trypsin digestion was more beneficial in this regard. That such cells were able to respond to LHRH in vitro was taken as evidence that these cells were not greatly damaged during isolation. However, there was still no evidence for hormone secretion by such cells in vivo, after transplantation. Experiment 3 was initiated to investigate whether pieces of pituitary would retain their endocrine function after transplantation, since such pieces had been shown to secrete a wide range of hormones in vitro, and to respond to stimulation by trophic hormones in culture (see Hopkins and Farquhar, 1973). However, these grafts were also apparently unable to secrete hormones after transplantation. Hypophysectomy of the recipients is not thought to have been contributing to these problems in any way, since the problem persisted when normal animals were also used in Experiment 3. No general conclusions could be drawn from this third experiment, about the usefulness of the testis as an immunologically privileged site over other recognised non-privielged sites, such as muscle. In the absence of detectable hormone secretion, no ready means was available to assess graft survival or function. It is possible that not enough time was allowed for grafts to recover from isolation and transplantation, and that this had affected hormone secretion by the grafts.

As discussed by Setchell (1978) the testicular capillaries appear to filter a fluid with a high concentration of protein. Testicular lymph in a ram has more than two-thirds as much protein as blood

plasma (Lindner, 1963; Wallace and Lascelles, 1964) which is much higher than lymph elsewhere in the body (except for the liver). It has also been found to be an important route of exit for conjugated steroids in the horse and pig testis (Setchell and Cox, 1982; Setchell et al, 1983). It is quite possible therefore that graft derived hormones may leave the testis in testicular lymph rather than in venous blood. Because it is so difficult to sample testicular lymph in the lymphatics in the spermatic cord of small animals such as the rat (see Morris and McIntosh, 1971), such samples were not available in this study.

The histology of the tissue grafts is very interesting. The presence of sperm in the tubules closest to the graft was unexpected given the lack of detectable hormone secretion in the blood. It suggests that local secretion by graft tissue may have been sufficient to allow continued development of one or more lines of germ cells. However, that Sertoli cells and other germ cells cannot be seen in these tubules makes it difficult to understand how such development might have occurrred. An alternative explanation is that insertion of the graft caused a localised pressure on surrounding tubules and may have caused a blockage in the tubule. This might account for the general lack of cells in the tubular epithelium. However, why sperm should survive in this situation is difficult to understand. This situation seems worthy of further investigation. Immunohistochemistry would have been useful in determining the likely role of hormone secretion in this situation. Nevertheless, it is interesting to note a number of literature reports which deal with tissue grafts of the anterior pituitary. In a number of previous reports, the importance of

neurosecretor stimuli for the anterior pituitary have been addressed. A number of workers have reported that transplanted anterior pituitary glands lose their functional capacity after transplantation into hypophysectomised animals, presumably due to a lack of secretomotor innervation. While Gardner and Hill (1935), Hill and Gardner (1936), May (1935, 1937) and Greep (1936) reported normal function for transplanted anterior pituitary tissue, Harris and Jacobsen (1952) re-examined these works and found a number of technical problems with the methods employed and questioned the validity of the approach of these authors. They went on to propose that graft failure might be due to the loss of contact of the graft with the hypophyseal-portal vessel, which might normally be associated with neural control of the anterior pituitary. Support for this hypothesis was found when they demonstrated that grafts of anterior pituitary placed into the subarachnoid space under the median eminance of the tuber cinereum became richly vascularised and remained viable for long periods, while grafts placed into the pituitary capsule did not survive so well. This would account for the lack of LH and FSH secretion noted in experiment 3 of the current study.

Prolactin however, should be readily detectable in anterior pituitary grafts even with the above hypothesis. Separation of the gland from the hypothalamo-portal system removes the prolactin-inhibiting factors which usually control the secretion of this hormone in the intact pituitary. Thus, the secretion of prolactin should increase considerably. Indeed, a recent report by Weber et al (1983) has in fact reported significantly elevated levels of Prolactin in the venous blood of rat testes containing pituitary grafts under the capsule. These authors also found significant levels of prolactin

in the testis containing the graft, and reported increased testosterone levels in tissue in the vicinity of the graft over the levels in the rest of the testis. This is not surprising given the gonadotrophic effect of prolactin reported by Nicoll and Bryant (1972), who proposed that prolactin may be important in the mammalian gonad in providing pools of precursors which can be mobilised by FSH and/or LH for the production of steroid hormones such as androgens in the testis. In experiment 3, the significant reduction of prolactin levels in normal rats following castration probably reflects the inverse relationship reported to exist between secretion of prolactin and gonadotrophins (see Grandison et al, 1977). The histological findings from experiment 3, of sperm in tubules around the grafts, may be due to local effects on androgen production by graft-derived prolactin as proposed by Weber et al (1983).

The discrepancy in blood hormone levels reported by Weber et al (1983) and those of the present study, is difficult to understand. It may be of consequence that Weber et al made their measurements at 100 days after transplantation, in comparison to the measurements at 21 days in the present study. Certainly, the pituitary is not the easiest tissue to work with. Not only is it relatively difficult to obtain in the first instance, but its care after isolation requires particular attention. Plasma concentrations of prolactin have been shown to change dramatically under various experimental conditions (Matthiej and Swarts, 1978), and there are problems with relying on prolactin levels as an indication of graft function after transplantation. Stress can considerably affect plasma prolactin levels, as can anaesthesia (Seggie and Brown, 1975). These problems would be of particular concern in intact animals bearing pituitary grafts since

the animals' own pituitary may affect measurements of any graft derived hormones. Such problems would have to be carefully addressed if further studies were to be performed with this tissue.

3.4. THYROID TRANSPLANTS

At this point I chose to investigate the use of another tissue for transplantation; one that could be more easily obtained, and one whose function could be more easily assessed in a variety of ways without necessarily terminating the graft. The thyroid gland was an obvious candidate. Because it concentrates iodine from the blood, its function can be readily and quantitatively assessed in situ by monitoring the uptake of administered radioactive iodine. It secretes a number of hormones (eg. Thyroxine, Tri-iodothyronine), the concentrations of which can be easily monitored in plasma, and it is a tissue which is readily accessible surgically. One lobe or the whole gland can be removed without serious problems for the donor, and certainly without causing the levels of mortality reached with hypophysectomy.

Given the problems encountered with the pituitary transplants, the basic aims of this project remained to be fulfilled. A routine transplantation protocol that was consistantly effective had to be developed, the immune privileged status of the rodent testis remained to be confirmed, and components of the interstitial region of the rodent testis that might be important in creating the proposed immune privileged status of this site had still to be examined.

In developing a routine transplantation protocol, it was decided

to use a single entire lobe of thyroid for transplantation. Entire recipients were used, and rather than measure hormone secretion from the grafts (which would be complicated by the presence of the animal's own thyroid), it was decided to use iodine accumulation as a measure of graft function in situ. Two experiments were performed in the first instance. Initially, the ability to transplant thyroid lobes from one rat into the testis of another rat was checked, and secondly, the usefulness of the testis as a transplant site was compared with that of the kidney capsule - a site used extensively by immunologists studying graft rejection (see Lafferty et al, 1975; Gose and Bach, 1984). A comparison of graft survival over time between these two sites was made in this second experiment with grafts being terminated at various times after transplantation.

3.4.1. Experimental Procedure

3.4.1.1. Animals

Male Porton rats were used in these experiments and maintained as described in 3.3.1.1. These animals were not inbred.

3.4.1.2. Experiment 4. Survival of thyroid allografts in the testis

Thyroid lobes were removed from three male donors under pentobarbitone anaesthesia as described in 2.3.1. and placed into sterile Dulbecco's PBS. Five male recipients (210 - 270 grams) were transplanted with one thyroid lobe into one testis as described in 2.3.7.2. Three weeks after transplantation, each animal was injected

with ^{125}Iodine and grafts removed 24 hours later. These procedures have been described in detail in 2.5.1.1. Transplanted testes were processed for histological examination as described in 2.14. following measurement of accumulated radioactivity in both grafted and control testes.

3.4.1.3. Experiment 5. A comparison of graft survival in the kidney and the testis

Thyroid lobes were removed from sixteen male donors as for experiment 4 above. Thirty-two male recipients (340 - 470 grams) were randomly allocated to four groups of eight animals. Each animal was transplanted with one thyroid lobe, and in each group half the animals received transplants into one testis and half received transplants under the capsule of one kidney as described in 2.3.7.1. and 2.3.7.2. At 1, 2, 4, and 8 weeks after transplantation, one group of animals was selected and injected with ^{125}Iodine and grafts removed 24 hours later as in experiment 4. Radioactivity was monitored in both grafted and nongrafted organs in each animal as detailed in 2.5.1.1. Grafted tissues were then processed for hisological examination as described in 2.14.

3.4.2. Results

3.4.2.1. Experiment 4. Survival of thyroid allografts in the testis

Four of the five animals grafted showed significant accumulation of iodine in the grafted testis compared to the control testis as indicated by a concentration difference of more than 4 times. This

measure of "significance" was adopted from the work of Lafferty et al (1975) who correlated iodine accumulation measurements with histological evaluation and found that a 4 fold or greater accumulation of iodine in the grafted organ over the control was indicative of a healthy graft. On histological examination, identifiable thyroid follicles containing colloid were found in the four animals with significant iodine accumulation. The fifth animal showed an iodine accumulation in the grafted testis of 3 times that of the control testis, and while histological studies suggested healthy tissue was present in the graft, it was not included as a successful graft under the above criteria. Figure 3.7. shows thyroid grafts in the testis at the time of transplantation (Figure 3.7.a) and at three weeks after transplantation (Figure 3.7.b). As was seen with the pituitary grafts, the centre of the thyroid graft has degenerated in this time, although healthy follicles are found around the periphery of the tissue mass. It is presumed that these are the follicles concentrating the iodine as discussed above. Figure 3.7.c shows a graft recovered from an animal six months after transplantation, and general regeneration of the central follicles of the graft has occurred.

3.4.2.2. Experiment 5. A comparison of graft survival in the kidney and the testis

As in experiment 4, graft survival was assessed under the guidelines of Lafferty et al (1975). The percentage of grafts surviving in the testis and kidney at 1,2,4,and 8 weeks after transplantation, as assessed by iodine accumulation measurements, is shown in Figure 3.8. Intratesticular grafts were apparently unable to

Figure 3.7. Thyroid allografts in the testes of adult rats: (a) at the time of transplantation, (b) three weeks after transplantation, and (c) six months after transplantation. While the central mass of the graft is necrotic at 3 weeks after transplantation, healthy follicles survive on the edge of the graft. By six months, the whole graft is reconstituted. Disruption of seminiferous tubules in (c) is due to physical disruption during tissue processing. X 80.

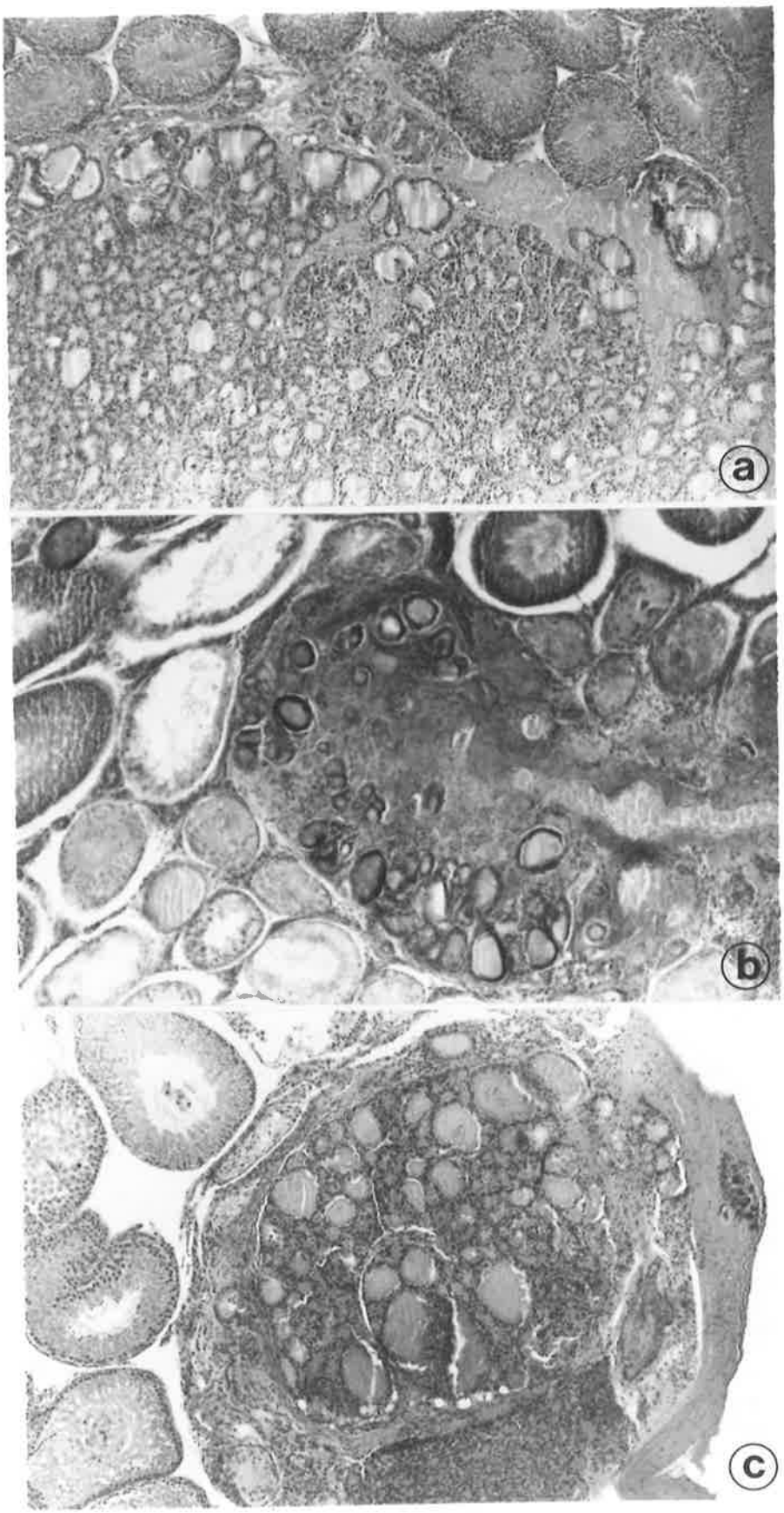
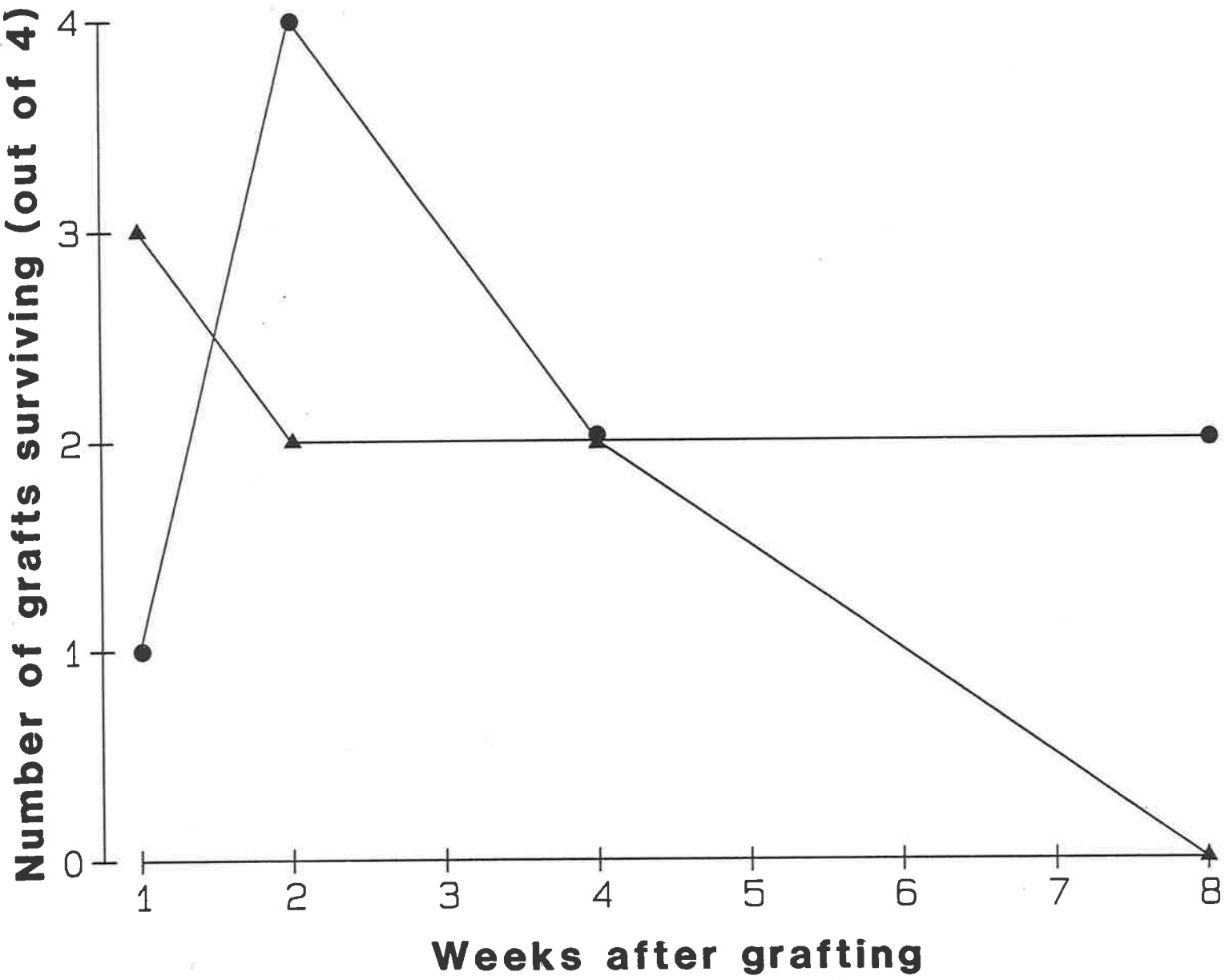

a
b
c

Figure 3.8. Number of surviving thyroid allografts in the testes (solid circles) and kidneys (solid triangles) of adult rats at 1,2,4, and 8 weeks after transplantation (n=4 animals per group). Survival was measured by accumulation of radioactive ^{125}Iodine, and a success recorded if the grafted organ was found to contain more than four times the amount of radioactivity measured in the contralateral, ungrafted organ.


Number of grafts surviving (out of 4)
0
1
2
3
4
1
2
3
4
5
6
7
8
Weeks after grafting

concentrate significant quantities of iodine at 1 week after transplantation, although all the testicular grafts were apparently concentrating iodine by week 2 of transplantation. It should be noted that all intratesticular grafts were accumulating iodine at 1 week after transplantation, but they did not meet the 4-fold criteria discussed earlier. There was apparently some mortality after this time, with only 50% of intratesticular grafts able to concentrate significant quantities of iodine by weeks 4 and 8 of the experiment. In contrast, while 75% of thyroid grafts placed under the kidney capsule were able to concentrate iodine at 1 week after transplantation, this ability rapidly declined and by 8 weeks after transplantation, no iodine accumulation could be registered in any thyroid grafts placed under the kidney capsule. Figure 3.9.a shows a thyroid graft under the kidney capsule at 1 week after transplantation, and Figure 3.9.b shows a similar graft at 8 weeks after transplantation. Rejection of the grafts is clearly demonstrated at 8 weeks with a massive lymphocytic infiltration. A close correlation was seen between the presence of follicles and the concentration of iodine.

Histology showed normal thyroid tissue in all intratesticular grafts at 1 and 2 weeks after transplantation. Figure 3.10.a shows a graft one week after transplantation. Necrosis of the central region has already occurred by this time, although healthy follicles are seen in the peripheral regions of the graft. By weeks 4 and 8, colloid-containing thyroid follicles were only found in those grafts that had also accumulated iodine, and Figure 3.10.b shows such a graft at 8 weeks. Note that there is not much difference between the 1 week and 8 week grafts in the testis, compared to the change seen in this

Figure 3.9. Thyroid allografts transplanted under the kidney capsule in adult male rats: (a) at 1 week, and (b) 8 weeks after transplantation. Note the healthy thyroid follicles at 1 week which have been destroyed by the massive lymphocytic infiltration by 8 weeks. Tissue grafts are indicated (T) to distinguish them from normal kidney tissue (K). X 80.

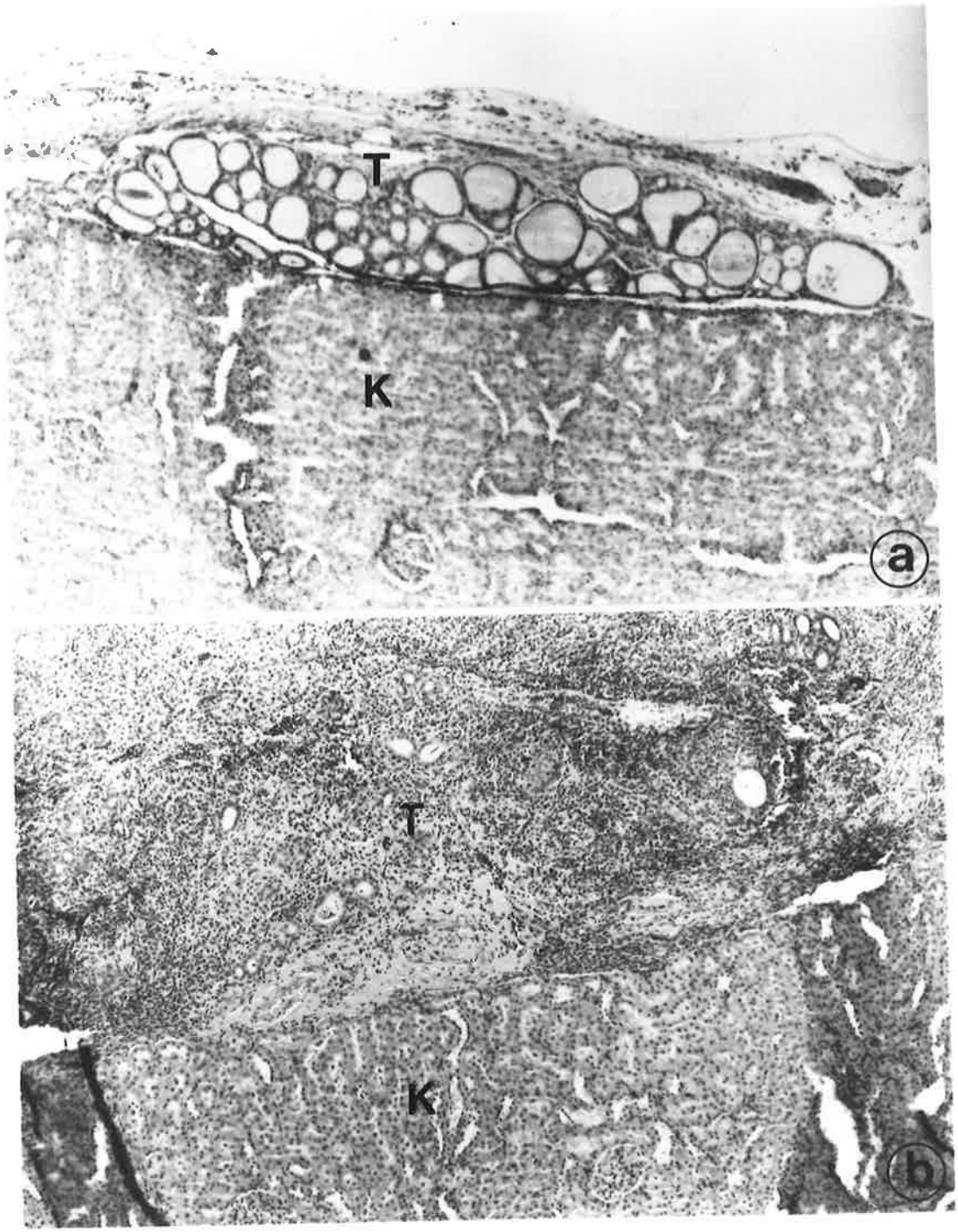
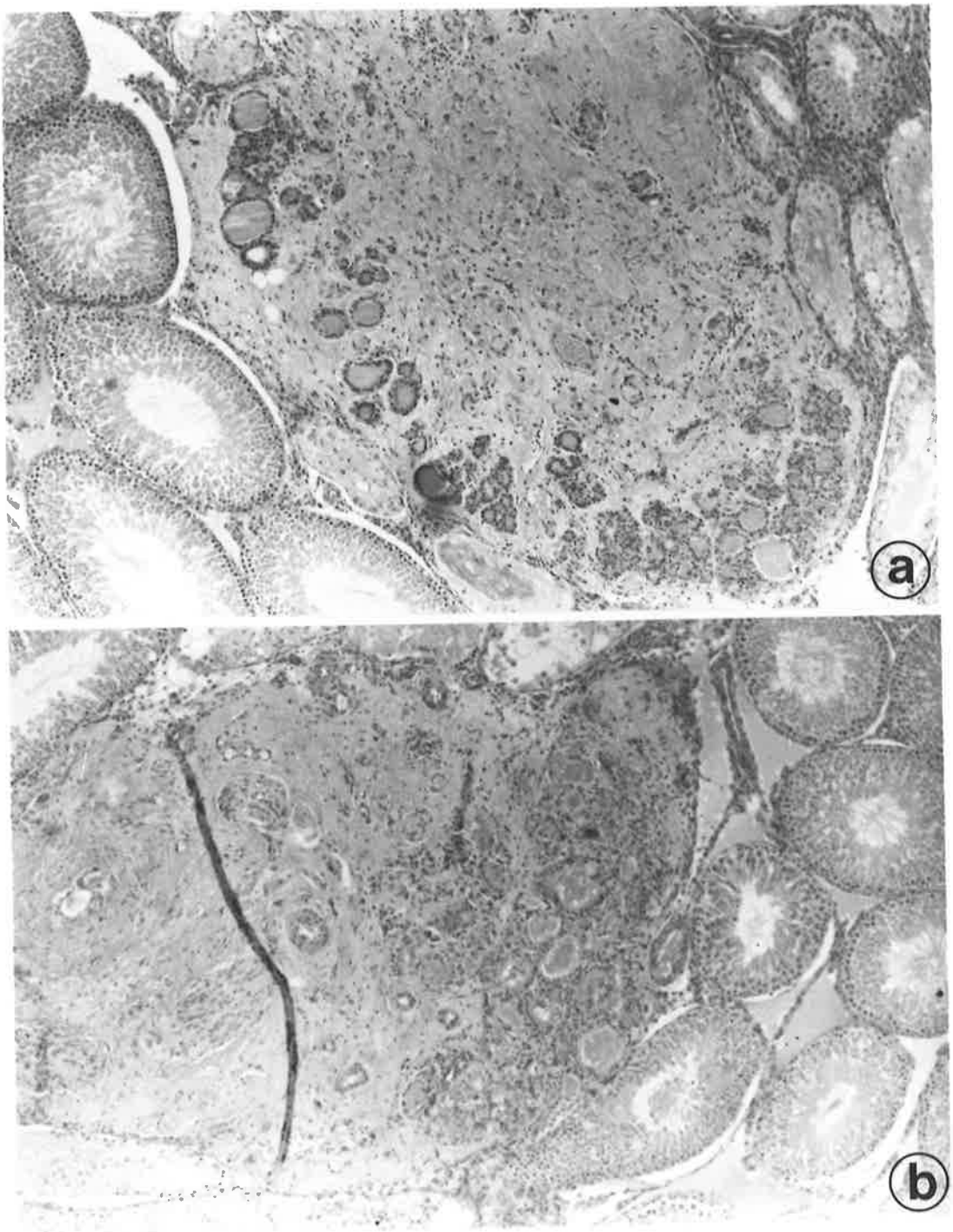

T
K
a
T
K
b

Figure 3.10. Thyroid allografts transplanted into the testis of adult male rats: (a) at 1 week, and (b) 8 weeks after transplantation. Necrosis of the central region of the graft has already occurred by 1 week, and no significant change is seen from this time to 8 weeks after transplantation. Healthy follicles are seen on the edge of the graft in both cases. A crease in the tissue section can be seen in the necrotic region of the graft in (b). X 80.


a
b

time in the kidney (Figure 3.9.).

3.4.3. Discussion

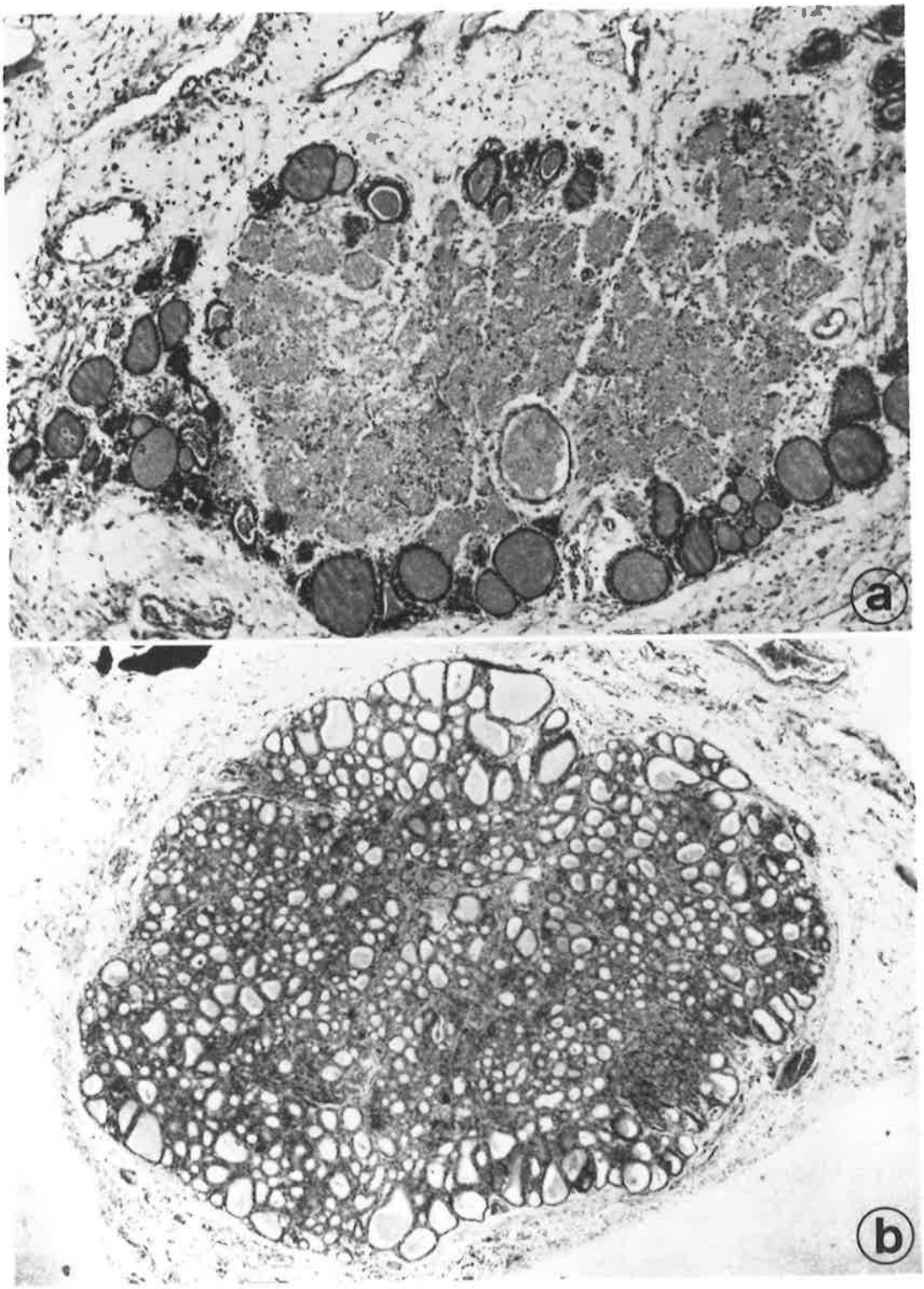
The results of these experiments demonstrated that the thyroid was a most suitable tissue for use in the proposed transplantation studies. It was easily obtained and transplanted, its survival was readily monitored in vivo, and grafts were easily identified in histological studies. A routine transplantation protocol was thus available.

The results of experiment 5 confirmed the previous reports that the testis possesses some degree of immunological privilege. Likewise, reports that the testis may not be completely privileged (see chapter 1) are also born out in the present study with the loss of some grafts over a period of 8 weeks. However, that grafts were continuing to concentrate iodine in the testis some 2 months after transplantation while those in the kidney were not is seen to indicate that this site does have an immunologically privileged status. Why thyroid grafts in the testis did not all accumulate iodine at 1 week after transplantation is difficult to understand. Lafferty et al (1975) reported that mouse thyroids were rapidly vascularised when placed under the kidney capsule, and this would not only be essential for graft survival, but also for radioactive iodine to reach the graft for accumulation. Thyroid grafts in the testis may not be sufficiently vascularised by the end of the first week after transplantation to be able to accumulate administered iodine. The lymphatic sinusoids of the rodent testis may provide sufficient nourishment for these grafts

during this time, and may maintain grafts until vascularisation is completed. However, free iodine should also be able to enter this lymphatic space and these grafts should thus have had access to sufficient iodine. That the histology of all intratesticular grafts was apparently normal at this time lends support to the suggestion that grafts may not have seen sufficient iodine at this time, and perhaps other factors were involved.

Comparison of the histology from the present experiments with that reported by other authors is of interest. Dempster (1957) has published pictures of thyroid autografts at 48 hours and 20 days after transfer to subcutaneous tissues and these are shown in Figure 3.11. In the present experiments, necrosis of the main mass of thyroid tissue was found within 1 week of transplant, and Dempster (1957) found this to have occurred within 48 hours in autografts as seen in Figure 3.11.a. According to Dempster, it is the remaining peripheral follicles that regenerate to reconstitute the tissue mass. It is interesting that he found this regeneration to have occurred within 20 days in subcutaneous tissue (3.11.b). This was not found in the testis in the present study even at eight weeks after transplantation, but was apparent by 6 months after transplantation (see Figure 3.7.). It is these findings that lend further support to the suggestion raised earlier that tissue regeneration and probably vascularisation of the graft are somehow impaired in the testis. No explanation can really be offered at this stage as to why this might be so. However, in a recent review on angiogenesis, Findlay (1986) has reported the angiostatic action of certain steroids when copresented with heparin. As discussed in chapter 1, mast cells are found in the testis, and are known to secrete heparin, and it may be that this allows certain steroids to

Figure 3.11. Thyroid autografts transplanted into subcutaneous tissues: (a) at 48 hours and (b) 20 days after transplantation. While the main mass of the graft has necrosed by 48 hours, there is virtual reconstruction of the lobe by 20 days. (a) X 95. (b) X 50. (from Dempster, 1957).


a
b

act as angiostatic factors in the testis and thus affect the regeneration of intratesticular grafts. Such a mechanism might also explain the natural prevention of vascular infiltration of the seminiferous tubules. These proposals require further investigation. They may also be of consequence for immune reactions in the testis. The presence of lymphocytes seen in the histological preparations, in or around successful and rejected tissue grafts, requires mention. Such populations have also been reported by Janney et al (1982) and Bobzien et al (1983) and it seems that the mere presence of lymphocytes around a graft cannot be assumed to mean that graft survival may be in jeopardy. Janney et al (1982) characterised the lymphocytes found around xenografts of pancreatic islets of Langerhans, and found that in successful grafts (where grafts maintained normoglycaemia in diabetic mice) the lymphocytes were largely suppressor lymphocytes and were likely involved in assisting graft survival. In rejected grafts however, the population were predominantly T-cytotoxic lymphocytes.

A number of authors have demonstrated that allograft immunogenicity can be reduced if the tissue to be transplanted is maintained for a period in organ culture (see Lafferty et al, 1975; Gose and Bach, 1984; Leuker and Sharpton, 1974; Lafferty et al, 1983). This is thought to be due to a reduction in the number of leukocytes carried in the transplanted tissue during the period of culture (Lafferty et al, 1983; Lafferty, 1984). While the process is certainly beneficial, there remains a degree of graft mortality. Leuker and Sharpton (1974) demonstrated significant survival of ovarian allografts following organ culture, with some 50% of cultured allografts still functioning 90 days after transplantation, compared

to total loss of all uncultured allografts by 35 days after transplantation. As seen in Figure 3.12., the results obtained in these studies, while over a slightly different time scale are similar to those found in experiment 5 of the present study (compare with Figure 3.8.). It is conceivable that the testis itself may provide some mechanism of removing, masking or inactivating graft-derived passenger leukocytes which allows the survival of uncultured allografts within its interstitial region. This would account for the similarity in the results discussed above, and might also explain the apparently incomplete immune privilege afforded grafts in this region. This proposal will be addressed in later sections of this thesis.

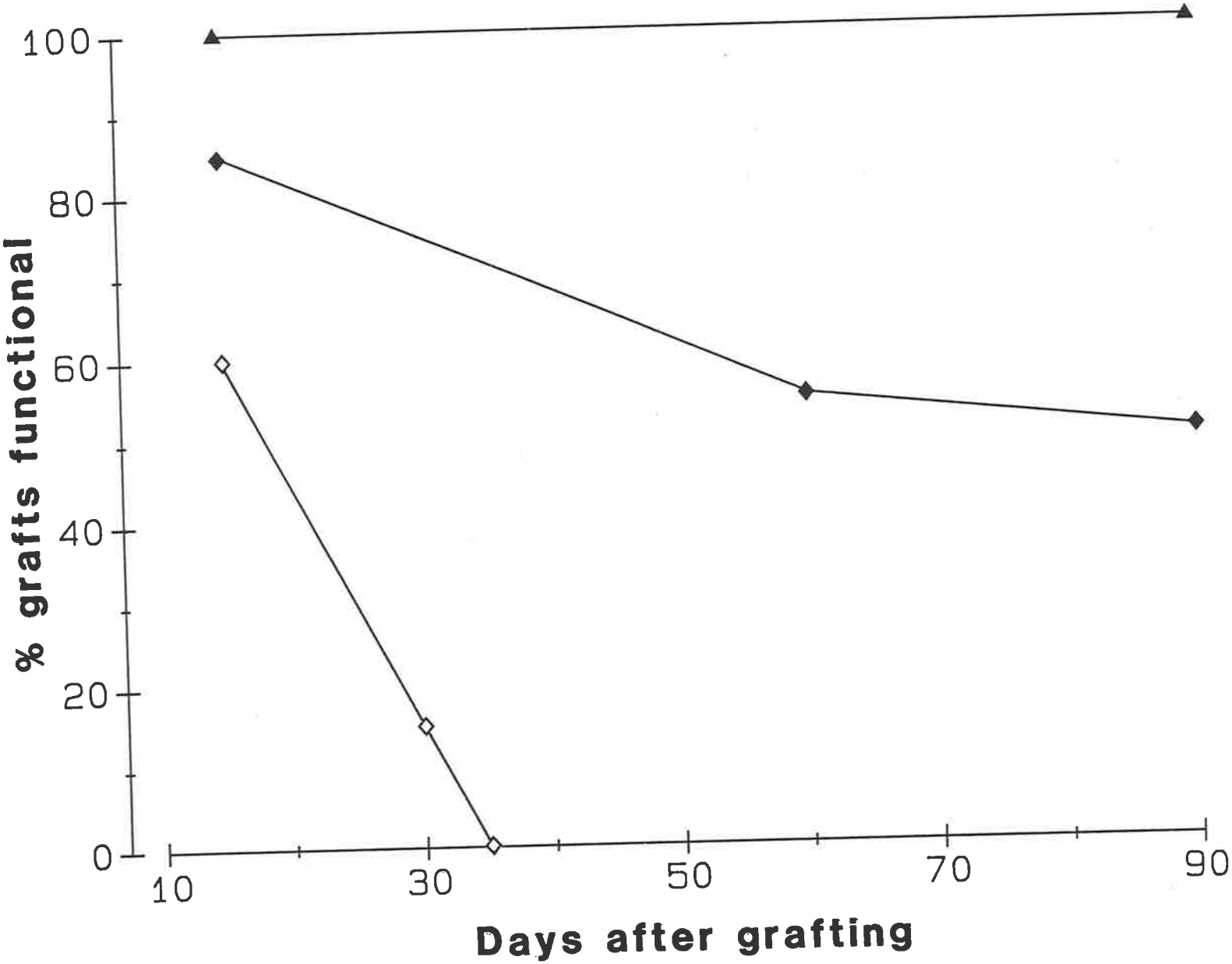
3.5. EXPERIMENTAL MANIPULATIONS OF THE TESTIS AND THEIR EFFECTS ON ALLOGRAFT SURVIVAL

In order to determine which components of the testis might be important in protecting allograft survival, a number of experimental techniques were used to alter various aspects of testicular function. The effects on thyroid allograft survival of cryptorchidism, ligation of the efferent ducts, and an increased lymph flow from the testis induced with human Chorionic Gonadotrophin, were tested. Graft survival in testes in which Leydig cells and/or macrophages had been specifically destroyed was also examined.

3.5.1. Efferent Duct Ligation

Ligation of the efferent ducts leading from the testis to the epididymis results in retention of the fluid which the testis secretes. The testis becomes distended and turgid, and ultimately the

Figure 3.12. Comparative survival of ovarian autografts (solid triangles), cultured allografts (solid diamonds) and non-cultured allografts (open diamonds) for up to 90 days after transplantation (from Leuker and Sharpton, 1974). Note the similarity in the pattern of graft survival of cultured ovarian allografts, to that seen with uncultured, intratesticular thyroid allografts presented in Figure 3.8.


100
80
60
40
20
0
% grafts functional
10
30
50
70
90
Days after grafting

germinal epithelium of the seminiferous tubules degenerates (Setchell, 1970). The blood-testis barrier is disrupted within 2-3 days after ligation, but reforms within 3 weeks (Setchell, personal communication).

The most prominent changes in the testicular milieu following efferent duct ligation were expected to arise from changes in factors contributed from the seminiferous tubules, and thus their importance in maintaining allograft survival could be assessed by this treatment.

3.5.1.1. Experimental Procedure

The efferent ducts of both testes in eight adult male rats (410 - 490 grams) were tied as described in 2.3.4. Thyroid lobes were obtained from donor males as detailed in 2.3.1. Four recipients were transplanted with one thyroid lobe into one testis at the time the efferent ducts were ligated, and the remaining four recipients were transplanted with one thyroid lobe three weeks after the efferent ducts were tied. Three weeks after transplantation, each animal was injected with ^{125}Iodine as described in 2.5.1.1. and the graft terminated 24 hours later. Transplanted testes were processed for histology as described in 2.14. following measurement of accumulated radioactive iodine in both grafted and control testes.

3.5.1.2. Results

All animals showed some iodine accumulation by the thyroid

grafts. In those animals transplanted at the time of efferent duct ligation, only three of the four showed significant accumulation (ratio > 4:1), while all animals grafted three weeks after duct ligation showed significant accumulation of iodine by the grafts. Figure 3.13.a shows the appearance of the testis at 3 weeks after efferent duct ligation. Spermatogenesis is clearly disrupted in this section, and large spaces are found between the tubules and interstitial tissue. Figure 3.13.b shows a thyroid graft in the ligated testis at 3 weeks after transplantation, and healthy follicles are clearly visible.

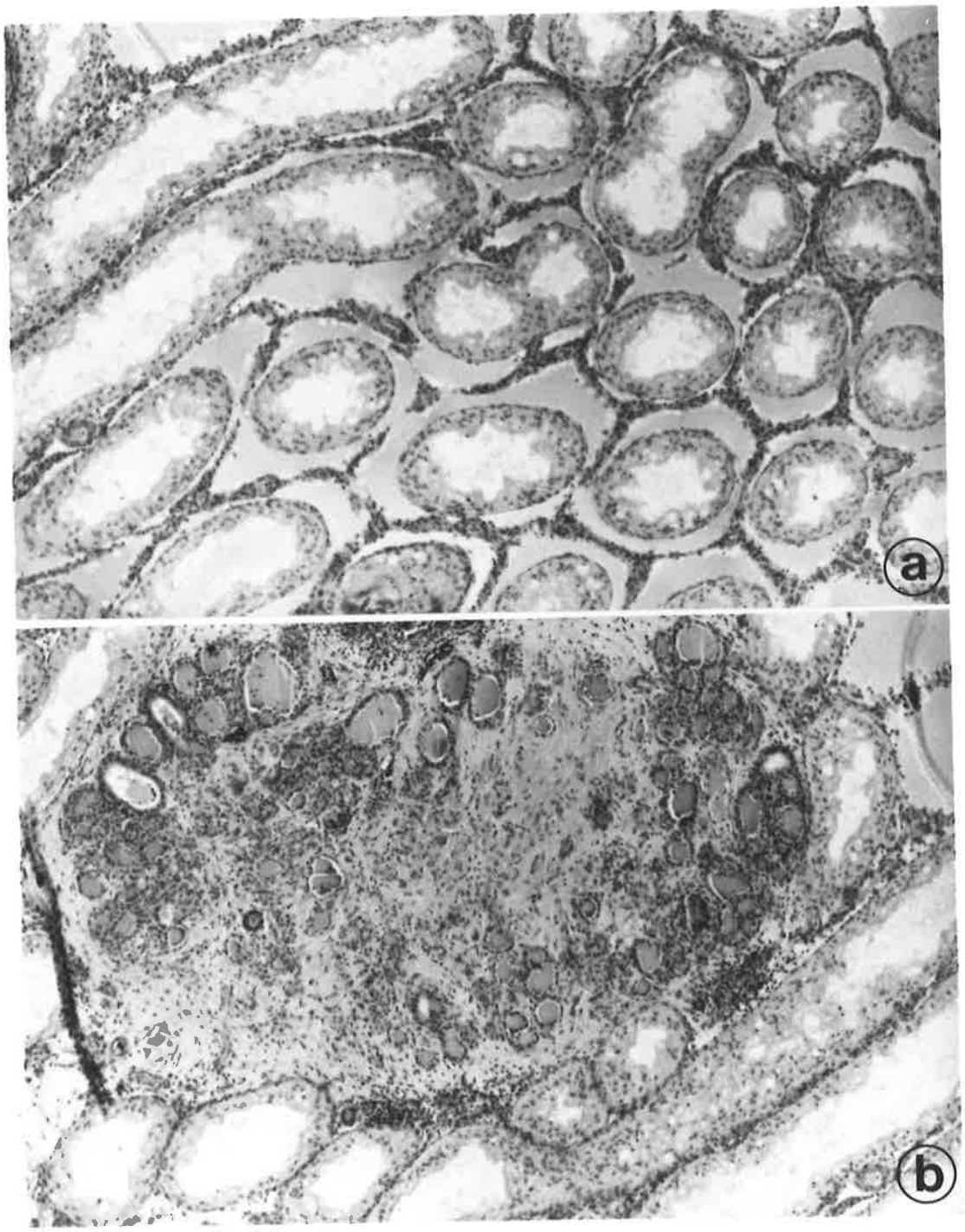
3.5.1.3. Discussion

Destruction of the germinal epithelium as induced by efferent duct ligation does not appear to have been detrimental for the thyroid allografts used in this study. The finding that the grafts survived when transplanted after distension and turgor had developed and after the tubular structure of the testis had collapsed (three weeks after duct ligation), indicates that the tubular compartment of the testis is unlikely to be involved in maintaining the immune privileged status of the testis.

3.5.2. Cryptorchidism

While cryptorchidism is a naturally occurring abnormality in animals normally found with scrotal testes, it is easily achieved experimentally in rats by placing the testis up in the abdominal cavity, and preventing its natural return to the scrotum.

Figure 3.13. (a) Testis of an adult rat 3 weeks after ligation of the efferent ducts. Spermatogenesis is clearly disrupted, and large extravascular interstitial spaces are seen between the tubules. (b) A thyroid allograft in the same testis shown in (a). Healthy thyroid follicles can be seen in the graft, which does not appear to have been affected by ligation of the testicular efferent ducts. X 80.


a
b

The most widely recognised effect of cryptorchidism on the testis is the induction of seminiferous tubule damage. Germ cells are rapidly depleted in the cryptorchid testis, although the Sertoli cells have been found only marginally altered morphologically. The Sertoli cells and some spermatogonia are the only cells remaining in the testis after long term cryptorchidism (Clegg, 1961, 1963). Sertoli cell function is affected by this treatment, since (within a few days of the procedure) ABP secretion is reduced to very low rates (Hagenas et al, 1977). The seminiferous tubules however are not the only testicular components to be affected. Marked changes in Leydig cell morphology and function also occur (see Sharpe, 1983). Leydig cell volume increases with hypertrophy of the cellular organelles involved in steroidogenesis (Kerr et al, 1979). Serum testosterone levels are often low to normal, despite these changes in Leydig cells together with increased circulating levels of LH. Sharpe (1983) concluded that the changes in Leydig cells were locally induced, and probably involved changes in the factors secreted by the Sertoli cells. Farrer et al (1985) reported impaired testosterone biosynthesis in the cryptorchid rat testis, and identified a number of enzymes involved in testosterone synthesis which were inhibited in the cryptorchid testis, but the animals studied were made cryptorchid soon after birth which may result in different effects to those observed in the testis that is made cryptorchid in the adult animal. Kerr et al (1979) have also indicated that damaged seminiferous tubules may metabolise testosterone to other metabolites such as oestradiol, and this might account for the apparent discrepancy between increased Leydig cell production of testosterone and normal or decreased plasma levels. Changes in the clearance rate of testosterone at the level of the

testis are unlikely to occur since blood flow changes have not been demonstrated in eutherian mammals as a consequence of cryptorchidism (Waites and Setchell, 1964; Setchell et al, 1966; Waites et al, 1968).

Cryptorchidism is another means by which alterations in tubular function can be achieved, although the aetiology of the disruption is quite different to that achieved with efferent duct ligation.

3.5.2.1. Experimental Procedure

Ten adult male rats (300 - 350 grams), maintained as described in 3.3.1.1. were rendered bilaterally cryptorchid by the method described in 2.3.3. Thyroid lobes were obtained from donor males as detailed in 2.3.1. Five recipients were transplanted with one thyroid lobe into one testis at the time the testis was placed in the abdomen, and the remaining five recipients were transplanted with one thyroid lobe into the testis three weeks after they were made cryptorchid. Three weeks after transplantation, each animal was injected with ^{125}Iodine as described in 2.5.1.1. and the graft terminated 24 hours later. Transplanted testes were processed for histology as described in 2.14. following measurement of accumulated radioactive iodine in both grafted and control testes.

3.5.2.2. Results

All animals showed significant accumulation of iodine in the grafted testes when compared to the ungrafted testis, and in all

cases, the ratio between both testes was greater than 4:1. There was no apparent difference between grafts transplanted at the time of placing the testis into the abdomen, and those grafted three weeks after the testis was placed in the abdomen. Colloid-containing thyroid follicles were found in all grafts. Histologically, the grafts and testes were very similar to those shown in Figure 3.13. after efferent duct ligation.

3.5.2.3. Discussion

These results suggest that the immune privileged status of the cryptorchid testis is as effective as that of the scrotal testis. Graft survival does not appear to be affected by increased temperature, or by the destruction of spermatogenesis seen in experimentally-induced cryptorchid testes. This is in agreement with the findings of Selawry and Whittington (1984) who found the cryptorchid testis to be a suitable site for pancreatic islet allografts (see also 3.3.).

3.5.3. Human Chorionic Gonadotriphin

Lymph flow from the rat testis is lower than in other mammals (Rat - 0.5 ul/g/h Setchell and Sharpe, 1981; Ram - 1 ul/g/h Lindner, 1963; Boar - 10 ul/g/h Setchell et al, 1983). This might possibly allow allograft antigens to be presented to the host immune system in a manner that would induce immune tolerance rather than rejection. Human Chorionic Gonadotrophin (hCG) has been shown to cause an accumulation of interstitial fluid in the testis beginning 8 to 12

hours after a single subcutaneous injection (Sharpe 1977a, 1979, 1980), caused by appreciable rises in vascular permeability in the testis, and accompanied by rises in lymph flow (Setchell and Sharpe, 1981). These changes, while apparent 8 hours after injection of hCG, peak around 24 hours post-injection. Subsequent studies have shown that the effect on vascular permeability is maximal at about 30 hours after injection of hCG and normal values are found again by 48 hours post-injection (Sowerbutts et al, 1986). If a protocol could be developed to maintain an increased lymph flow over a number of weeks, the importance of the rate of lymph flow for allograft survival could be assessed.

3.5.3.1. Maintenance of increased lymph flow with hCG

HCG leads to a down regulation of its receptors on the Leydig cells (Sharpe, 1976; Hsueh et al, 1976) and a reduced androgenic response to second and subsequent injections if given during the 48 hours following an initial hCG injection (Hsueh et al, 1977). It was therefore necessary, initially, to investigate the effect of repeated injections of hCG on testicular lymph flow, interstitial fluid volume and vascular permeability in adult male rats, in order to determine if an increase in lymph flow could be maintained over a greater period of time.

3.5.3.1.1. Experimental Procedure

Adult male rats (280- 570 grams) were maintained as described in 3.3.1.1. In the first experiment, four groups of ten rats received

daily subcutaneous injections of 50 i.u. hCG as described in 2.6. for either 1, 2, 3 or 4 days. Ten uninjected rats served as controls with measurements of vascular permeability, lymph flow and interstitial fluid volume being made 20-24 hours after the last hCG injection (see 2.10., 2.11., 2.12.). In a second experiment, four groups of five animals received either one dose of 50 i.u. hCG or 2 or 3 similar doses with either 48 or 72 hours between injections; measurements of albumin clearance were made 24 hours after the last hCG injection (see 2.12.).

3.5.3.1.2. Results

3.5.3.1.2.1. Effect of daily doses of hCG

The results show a marked response of ^{125}I-hSA spaces and half-time of clearance of ^{125}I-hSA 20 hours after the first hCG injection, indicating that there were increases in vascular permeability and lymph flow (Table 3.4.). While the ^{51}Cr-EDTA spaces tend to increase over the treatment period, these changes were not significant. Albumin clearance rates did not remain elevated after the first day of treatment and in fact fell significantly below control values by day 3 of treatment, returning to control values by day 4. In contrast to the original method of Setchell and Sharpe (1981), the values used to calculate lymph flow were obtained from two different groups of animals. The values presented are thus calculated on mean values only and standard errors cannot be determined. However they suggest that lymph flow was also elevated only on day 1 of treatment whereas vascular permeability peaked at 24 hours and then slowly

Table 3.4. Measured changes in one-hour distribution volume or 'space' of Cr-EDTA and albumin, half time of albumin clearance from the testis and the calculated lymph flow and vascular permeability after repeated daily injections of hCG (50 i.u.) (Means ± SEM, 5 animals per group).

Days of hCG treatment	Testis weights (g)	Distribution Volumes Cr-EDTA (µl/g)	Albumin (µl/g)	Half-time of Albumin clearance (min)	Lymph flow (µl/g/min)	Vascular permeability (µl/min/g)
0	1.30 ± 0.04	198.95 ± 12.19	34.90 ± 2.95	208 ± 23	0.66	0.47 ± 0.07
1	1.30 ± 0.03	237.50 ± 25.54	82.98 ± 8.61*	65 ± 13*	2.53	1.41 ± 0.30*
2	1.35 ± 0.04	239.14 ± 22.56	58.53 ± 4.75*	187 ± 6	0.89	0.85 ± 0.14*
3	1.16 ± 0.05	334.49 ± 31.65	58.70 ± 3.42*	540 ± 31*	0.51	0.82 ± 0.11*
4	1.13 ± 0.05	288.63 ± 11.11	48.52 ± 2.06*	187 ± 32	1.07	0.66 ± 0.04
L.S.D.	n.s.	n.s.	14.59	62.86		0.32

Analysis of variance and covariance (against testis weights) was used to detect significant treatment differences, and where appropriate the Least Significant Difference value (L.S.D., 5% level) is listed (n.s. indicates no significant difference). Values differing significantly from the control value are indicated (*).
Values for Lymph Flow are calculated from the results of two different treatment groups. Consequently group means have been used in calculations and standard errors and levels of significance cannot be determined.

returned towards normal.

3.5.3.1.2.2. Effect of doses of hCG every 2 or 3 days

Elevated clearance rates of albumin from the testis after the second and third doses of hCG were seen if the frequency of the injections was reduced from 24 hours to 48 hours or 72 hours (Table 3.5.).

3.5.3.1.3. Discussion

The present results on the effect of one injection of hCG on albumin space and albumin clearance confirm the observations previously reported by Setchell and Sharpe (1981) and Sowerbutts et al (1986). Previous studies also found an increase in interstitial fluid volume at 20 hours after a single hCG injection (Setchell and Sharpe, 1981). While the results obtained in the present study show a similar trend (^{51}Cr-EDTA space, Table 3.4.), none were found to significantly differ from control values, but the control values for the ^{51}Cr-EDTA space are higher in the present study than those found by Setchell and Sharpe (1981), and this may contribute to the lack of significance. No explanation can be offered for this difference.

Following repeated administration of hCG as used in the first experiment, there was a sustained elevation in albumin space and vascular permeability, although both tended to return towards control, and at no time were the effects as great as those following the first injection. By comparing these results with the effects of a single

Table 3.5. Half-time of clearance of albumin from the testis 24 hours after one dose or the last of a series of repeated doses of hCG with intervals of 48 or 72 hours between doses (Means ± SEM).

Days of treatment with hCG	Albumin clearance ($T_{1/2}$, min)
0	208 ± 23+
1	65 ± 13+*
1 and 3	65 ± 1*
1 and 4	107 ± 2*
1,3 and 5	88 ± 1*
1,4 and 7	86 ± 3*

+ from Table 3.4.

* All values are significantly ($P < 0.001$) less than the control value, the values in lines 4 and 5 are higher than line 2 ($P < 0.001$ and $P < 0.01$ respectively).

injection of hCG (Sowerbutts et al, 1986) it appears that second and subsequent injections were without effect on these parameters. Sowerbutts et al (1986) reported an albumin space of 53 ± 3.17 at 48 hours after a single injection of hCG which is not significantly different from the value in the present study of 58.53 ± 4.75 at 48 hours after the first and 24 hours after the second hCG dose (see Table 3.4.). The failure of interstitial fluid volume to increase significantly over the period of treatment as reflected by the ^{51}Cr-EDTA space, despite significant changes in vascular permeability, suggests that changes in vascular permeability were accompanied by concommitant changes in lymph flow. Why albumin clearance should be so dramatically reduced by day 3 of treatment is difficult to understand. Vascular permeability is still increased at this time, and presumably other factors may also be involved in regulating lymph flow and albumin clearance from the testis.

If injections are administered every 48 hours or 72 hours (second experiment) rather than every 24 hours, the response by the testis to each dose of hCG, in terms of albumin clearance (reflecting lymph flow in part) is similar to that in animals receiving a single dose. This means that the loss of response found in animals receiving daily injections of hCG can be fully recovered within 48-72 hours after the hCG injection. Setchell and Sharpe (1981) proposed that the primary effect of hCG in the testis is an indirect effect on vascular permeability with changes due to certain substances secreted by the testis in response to hCG. Steroids, prostaglandins and protein synthesis have not been found to have any influence over the effect of hCG on hormone uptake or interstitial fluid volume in the rat testis

(Veijola and Rajaniemi, 1985; Sowerbutts et al, 1986). However these authors have recently demonstrated the inhibitory effect of an antagonist to 5-hydroxytryptamine on albumin space increases after hCG treatment in the rat testis which may implicate mast cells in the actions of hCG on vascular permeability in the testis. If secondary substances are responsible for the increased vascular permeability after hCG administration, the repeated hCG doses may have affected production and/or release of these substances. Setchell and Rommerts (1985) have found that the hCG-induced increase in permeability of testicular blood vessels is completely abolished by pre-treatment with ethane dimethane sulphonate (EDS), a compound which eliminates the Leydig cells from the testis (Molenaar et al, 1985). However, an effect of EDS on other cell types has not been excluded. The close association of Leydig cells with testicular macrophages (Niemi et al, 1986) and mast cells (see Nistal et al, 1984; Maseki et al, 1981) may also be important for the actions of hCG, and would also be affected by treatment with EDS.

A reduction in testicular binding of hCG within 24 hours of treatment has previously been demonstrated (Hsueh et al, 1976, 1977; Sharpe, 1976) and this is related in part to loss of LH (hCG) receptors. There is also a large decrease in the capacity of the testis to secrete testosterone when stimulated again at this time (Sharpe, 1977b) and the two events may be related. The loss of LH receptors persists for long periods and is not maximal until 2 days after treatment (Hseuh et al, 1977). While the vascular response may be mediated via hCG receptors, the results from the present study show that a second hCG dose at the time of maximal receptor loss produces

the anticipated increase in vascular permeability. This suggests that the normal numbers of hCG receptors on the Leydig cell are not necessarily required for the vasoactive actions of hCG in the testis. It is interesting to note that Bergh et al (1986) and Widmark et al (1986) have recently presented evidence which implicates leucocytes in the hCG induced changes in testicular vascular permeability.

3.5.3.2. Effect of increased lymph flow on allograft survival

Having established that injections of hCG every second day maintained an increased lymph flow from the testis (as reflected by albumin clearance rates), the effect of this treatment on thyroid allograft survival in the testis was investigated.

3.5.3.2.1. Experimental Procedure

Twenty adult male rats (300 - 420 grams) were maintained as described in 3.3.1.1. Thyroid lobes for transplantation were obtained from adult male donors as described in 2.3.1. Recipients were randomly allocated to four groups of five animals. Two groups received one thyroid lobe transplanted into one testis, while the other two groups did not receive transplants. One of transplanted groups and one of the non-transplanted groups received no further treatment. The other transplanted group received subcutaneous injections of 50 i.u. hCG every second day as described in 2.7., as did the other untransplanted group. HCG injections were continued for three weeks after transplantation. Three weeks after transplantation and 24 hours after the last hCG injection, all transplanted animals were injected with

^{125}Iodine as described in 2.5.1.1. Twenty four hours later, testes were removed, and iodine accumulation by the intratesticular grafts was monitored. Grafts were then processed for histological examination as described in 2.14. At the time of graft removal, clearance of radioactive iodinated albumin from the testis was monitored in both groups of the non-transplanted animals as described in 2.12.

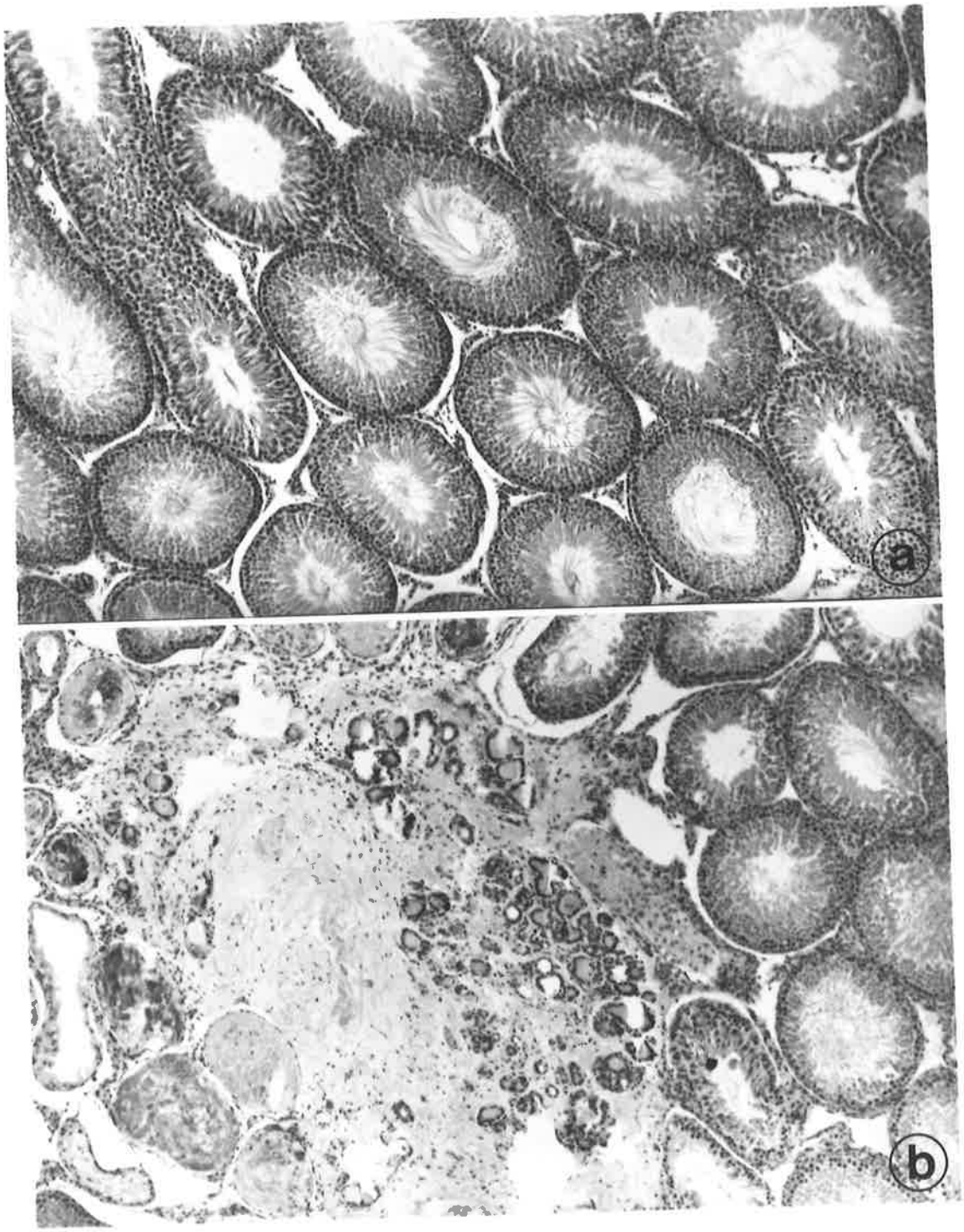
3.5.3.2.2. Results

The half-time of clearance of albumin from the testis in hCG injected animals was 56% of that in the uninjected control animals (108 $\pm$ 7.35 min : 192 $\pm$ 19.6 min, $P < 0.01$ - students t-test)

All transplanted animals showed significant iodine accumulation in the grafted testis when compared to the control testis (ratio > 4:1). There was no significant difference between graft survival as measured by the iodine ratio in the transplanted-control group and the transplanted-hCG injected group (4.43 $\pm$ 0.32 : 4.67 $\pm$ 0.52)

Histology showed colloid-containing follicles in all grafts, irrespective of treatment. Grafts in untreated animals were no different to those shown previously in Figure 3.14. As seen in Figure 3.14.a, hCG treatment did not alter the morphological appearance of the testis, which was very similar to that seen in control animals. Likewise the grafts were not greatly different to those of control animals, and Figure 3.14.b shows a graft after 3 weeks of hCG injections.

Figure 3.14. (a) Section of the testis from an adult rat injected subcutaneously with hCG every second day for 3 weeks. The appearance of the testis is no different to control testes. (b) A thyroid allograft in the same testis shown in (a). Healthy thyroid follicles can be seen in the graft, which does not appear to have been affected by hCG treatment. X 80.


a
b

3.5.3.2.3. Discussion

By measuring the half-time clearance of albumin from the testis and the volume of interstitial fluid (one hour Cr-EDTA space), a quantitative measure of lymph flow from the testis can be made (see 2.12.). However, half-time clearance of albumin alone is indicative of the state of lymph flow (see Table 3.4.). The majority of albumin injected into the testis leaves via the lymphatics (Setchell and Zupp, unpublished observations) and an increased clearance of albumin from the testis, with a constant or increased interstitial fluid volume, must reflect an increase in lymph flow. In the animals injected with hCG for 4 consecutive days there was no significant change in interstitial fluid volume, although the values do appear to have increased somewhat over controls. While such measurements were not made in animals injected with hCG every second day for two weeks, it was assumed that interstitial fluid volume did not fall during this time. Thus, the increased clearance of albumin from the testis in these animals is likely to reflect an increased lymph flow, and provides confirmation of the effectiveness of treatment. It was also assumed that the transplanted animals receiving the same hCG treatment would also have shown this response had they been tested (iodine administered to monitor graft survival prevented this). Since the survival of grafts in the hCG injected animals was no different to that in the uninjected animals, it appears that an increased lymph flow is not detrimental to graft survival. It is therefore likely that the slower rate of lymph flow in the rodent testis compared to that found in other mammals and other regions of the body, is not of importance in providing the immunologically privileged environment in

the rodent testis.

3.6. IMPORTANCE OF LEYDIG CELLS AND/OR MACROPHAGES FOR ALLOGRAFT SURVIVAL IN THE TESTIS

Leydig cells spontaneously adhere to lymphocytes and non-specifically suppress proliferation of mitogen-stimulated and allo-antigen stimulated lymphocytes in vitro (Born and Wekerle, 1982). Androgens and other steroid products of these cells have been shown to be immunosuppressive (Clemens et al, 1979; Roubinian et al, 1977). Consequently, the Leydig cell has been proposed as a likely contributor to local immunosuppression in the testis (Head et al, 1983c; Head and Billingham, 1985).

The drug ethane-dimethyl-sulphonate (EDS) has been used extensively in recent years to specifically destroy Leydig cells in rodents (Morris et al, 1986; O'Leary et al, 1986; Molenaar et al, 1985). While serious and sometimes fatal side effects occur in other species (eg. pigs die after EDS administration, Dr. C.J.G. Wensing, Dept. Veterinary Anatomy, State University Utrecht, The Netherlands, personal communication), no other effects have been noted in the rat.

It was of interest to examine allograft survival in animals in which Leydig cells had been destroyed by EDS.

However, a number of other cells are found in the interstitial tissue of the testis, and of particualar interest are the macrophages (see Niemi et al, 1986). While these cells do have phagocytic activities, they cannot be assumed to be the same as other roving macrophages of the immune system. They are resident in the testis, and as discussed in the introductory review (chapter 1) are found in close

association with Leydig cells. They have even been proposed as Leydig cell precursors in the rat (Clegg and MacMillan, 1965a). The possible role of these macrophages in providing the immune privilege in the rat testis has not been previously examined. Their common contamination of Leydig cell cultures might contribute to the reported immunosuppressive effects of Leydig cells in vitro, discussed above.

Hovatta et al (1986) have reported the effects of silica-induced damage to macrophages in the testes of rats. Allison et al (1966) and Oehler et al (1978) have previously demonstrated the cytotoxic effect of intraperitoneal injections of silica on macrophage activity, with associated loss of natural killer activity. However, Hovatta et al (1986) reported that testicular macrophages were apparently destroyed by intratesticular injections of silica, although the evidence was not conclusive. It was felt warranted however, to include an investigation of the effects of intratesticular injections of silica on allograft survival.

3.6.1. Experimental Procedure

Twenty eight adult male rats (350 - 480 grams) were maintained as described in 3.3.1.1. Thyroid lobes for transplantation were obtained from adult male donors as described in 2.3.1. In an initial trial, two groups of four animals were transplanted with one thyroid lobe into one testis, with one group receiving intraperitoneal injections of EDS as described in 2.8., and the other group receiving injections of vehicle only (DMSO/water). In the second experiment, recipients were randomly allocated to one of four treatment groups with five animals in each group, and again transplanted with one thyroid lobe into one

testis. One group remained untreated as the control group, one group was injected with EDS as described in 2.8., one group was injected with silica into the testis to be grafted, as described in 2.9., and the final group received both intraperitoneal EDS and intratesticular Silica. Silica injected animals were injected into the ungrafted testis with a similar volume of saline vehicle to monitor the effect of this technique on the testis. All animals were transplanted 3 days after treatment, and grafts terminated 3 weeks later. Animals were injected with ^{125}Iodine 24 hours prior to graft removal, as described in 2.5.1.1. After examining iodine accumulation in both testes of all animals, testes were placed in Bouins fixative and processed for histological examination.

3.6.2. Results

Graft survival was assessed by the 4:1 ratio rule of Lafferty et al (1975) discussed in 3.4.2.2. Results are presented in Table 3.6., grouping results of both experiments. One control animal in the initial trial did not survive the experiment, and one of the animals in the second experiment receiving both EDS and intratesticular Silica was rejected at the time of graft removal because the ungrafted, saline injected testis had regressed considerably, and could not be used as a control.

When graft survival in all EDS treated animals is compared to that in all non-EDS treated animals, graft survival was significantly reduced by EDS ($P < 0.01$). Such comparisons of survival in all Silica treated versus non-Silica treated shows no significant difference.

Table 3.6. Effect of EDS and/or Silica on thyroid allograft survival.

Treatment	Graft Survival Success	Fail	
Control	7	1	
EDS	3	6	a
Silica	3	2	
Silica + EDS	0	4	a
Combined non-EDS controls	10	3	
Combined EDS treatments	3	10	b
Combined non-Silica controls	10	7	
Combined Silica treatments	3	6	

a - significantly different from Control ($P < 0.05$)

b - significantly different from Combined non-EDS controls ($P < 0.01$)

(Significance tested in 2x2 contingency tables with X^2 analysis as described by Finney (1948) and Latscha (1952))

Three weeks after EDS administration, tubules appear relatively healthy Figure 3.15.a. The interstitial region is grossly underpopulated and the predominant cell type is the testicular macrophage (Figure 3.15.b). Tissue grafts are significantly infiltrated by lymphocytes as seen in Figure 3.15.c and no healthy follicles are obvious. At greater magnification however, the odd follicle remnant is seen (Figure 3.15.d). In the grafted testis, while the immune reaction is concentrated around the graft, there does appear to be some damage to tubules surrounding the graft, and the interstitial tissue in the vicinity of the graft also contains a significant number of lymphocytes (Figure 3.15.c). This is not the case in other regions of the testis (Figure 3.15.b).

Silica injections into the testis appear to have disastrous effects at the site of injection. Figure 3.16.a shows an immersion-fixed, saline injected testis, and healthy tubules at different stages of spermatogenesis are clearly seen, in association with relatively normal interstitial tissue. Figure 3.16.b shows the site of a silica injection into the testis. Spermatogenesis has been completely abolished in what remains of the seminiferous tubules, and the interstitial tissue appears to be infiltrated by large numbers of lymphocytes. This treatment has not affected graft survival however, and as seen in Figure 3.16.d the graft is easily identified amongst this mess of damaged tissue, with healthy colloid-containing follicles. It appears that the damage is localised to the site of injection, because the rest of the testis appears quite unaffected by the treatment. As seen in Figure 3.16.c, there is a clear deliniation of the damaged region and normal testis tissue.

The combined use of EDS and Silica has produced a combination of

Figure 3.15. Sections from the testis of an adult rat 3 weeks after treatment with EDS, and transplantation of an intratesticular thyroid allograft. (a) Seminiferous tubules are relatively healthy, and some contain sperm. X 80. The interstitial region is grossly underpopulated (b) and the predominant cell type is the testicular macrophage. Some regenerating Leydig cells are apparent. X 320. (c) The thyroid allograft has been destroyed by infiltrating lymphocytes, and no healthy follicles can be seen. This reaction also appears to have spread into the surrounding interstitial tissue. X 80. (d) At greater magnification, some follicle remnants are obvious (F). X 320.

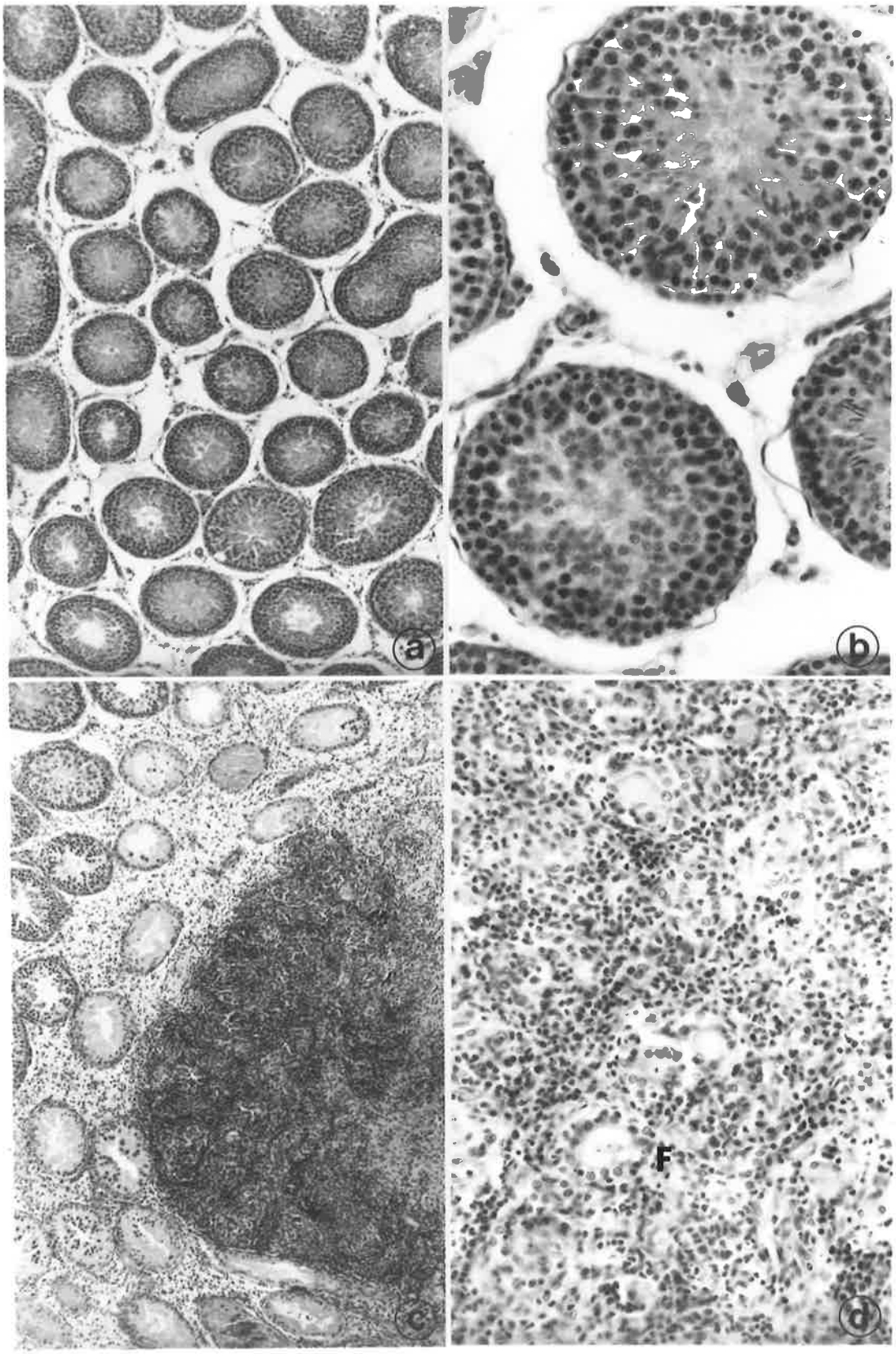
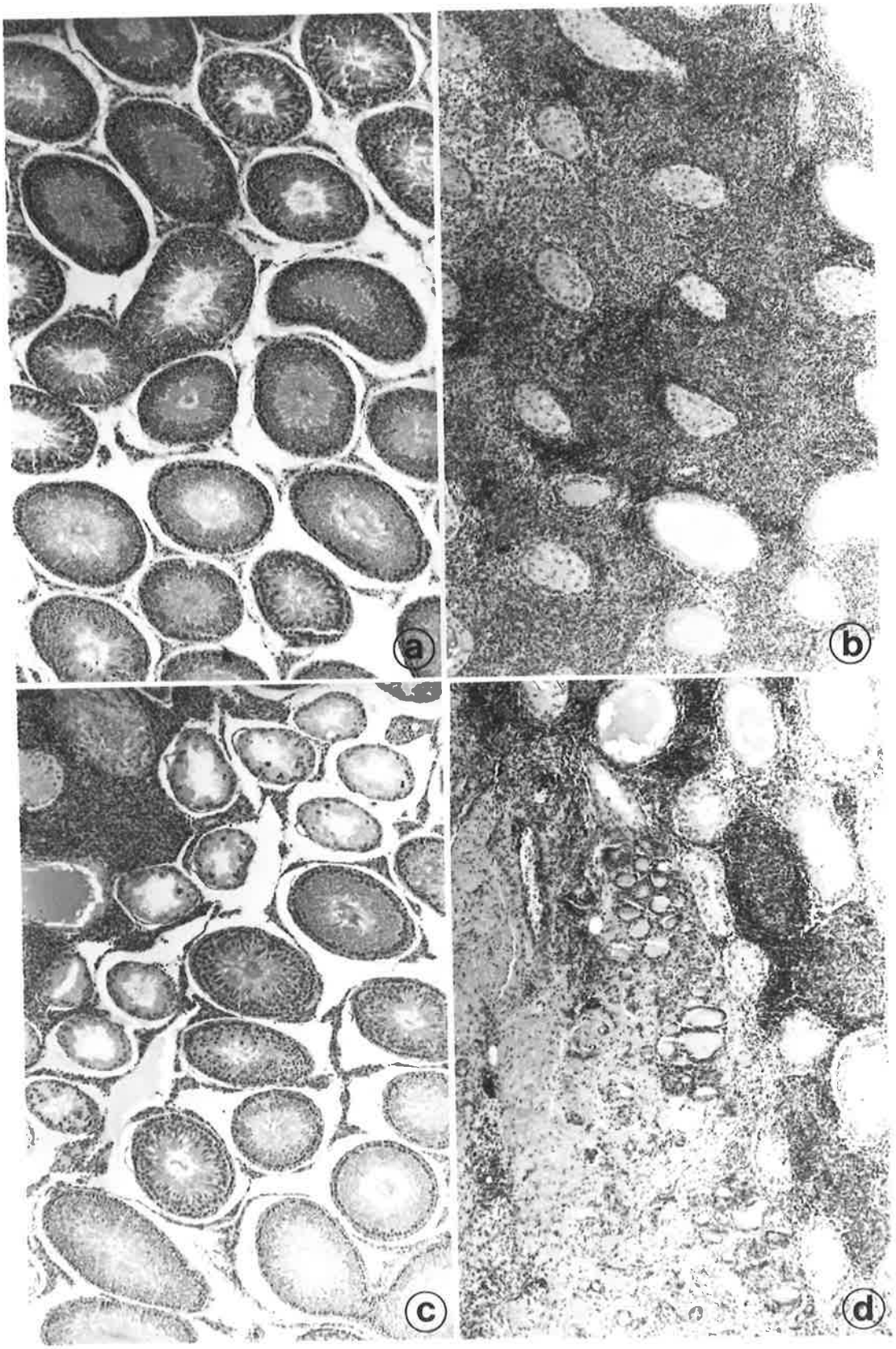

a
b
c
F
d

Figure 3.16. Sections from the testes of an adult rat 3 weeks after an intratesticular injection of Silica, and the transplantation of an intratesticular thyroid allograft. (a) Control section from a saline injected testis showing healthy tubules and interstitial tissue. (b) Site of injection of Silica particles into the testis. Spermatogenesis has been completely abolished in what remains of the seminiferous tubules, and the interstitial tissue is heavily infiltrated with lymphocytes. (c) The reaction to Silica is very specific, and clearly delineated. Tubules in other regions of the testis do not appear affected by the treatment. (d) Likewise, the thyroid allograft appears unaffected, despite being placed in the region damaged by Silica. Healthy thyroid follicles are seen in the peripheral regions of the graft. X80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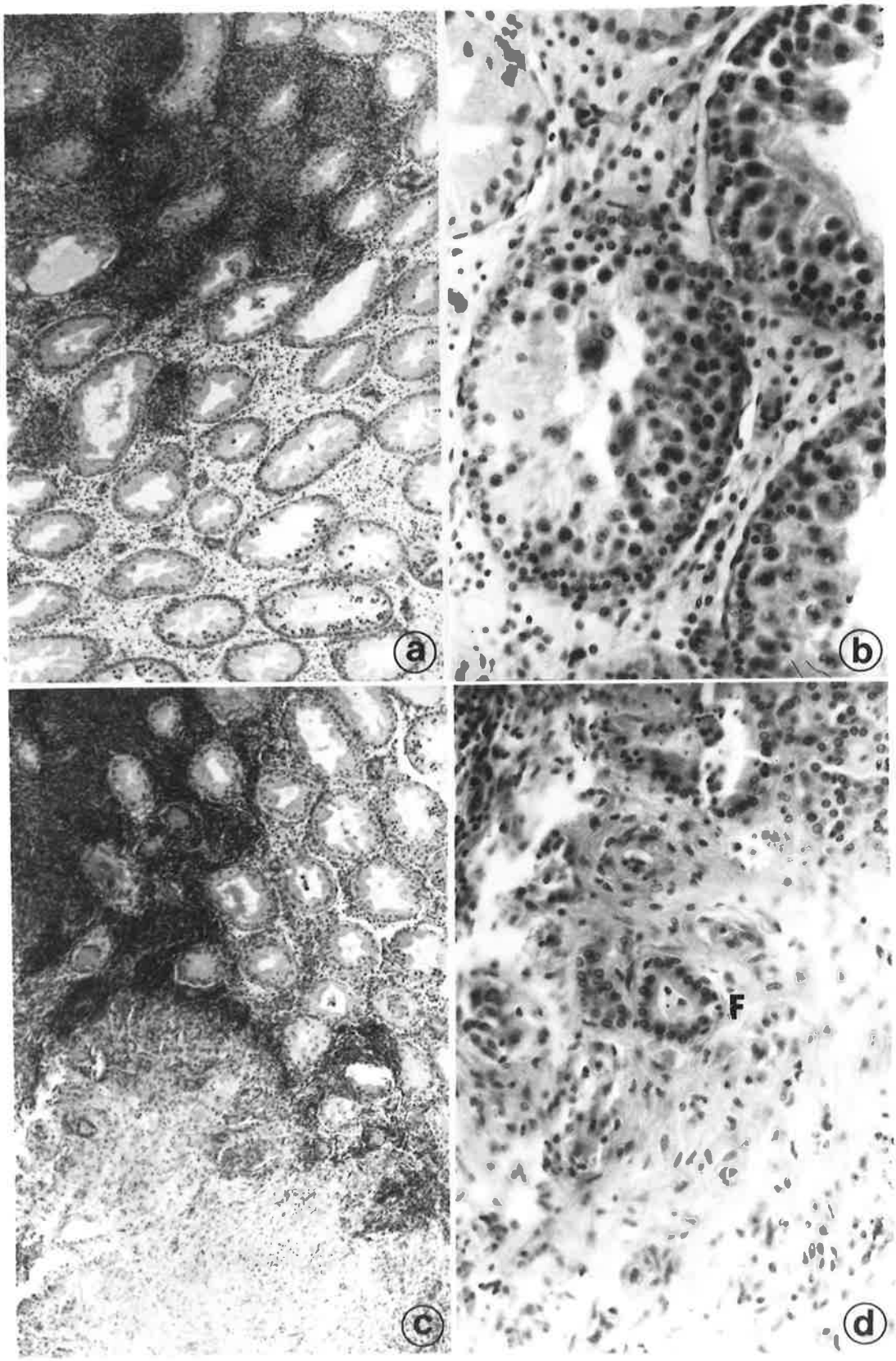
c
d

the effects seen when these treatments are used individually. The localisation of the silica-induced damage is again clearly evident in Figure 3.17.a and 3.17.c. The lack of Leydig cells in the interstitial region and the atypical appearance of this tissue after EDS treatment is again seen in Figure 3.17.b. The graft is seen in Figure 3.17.c, and while the severity of the immune response seen when EDS was used alone (see Figure 3.15.) is not obvious in this section, no healthy thyroid follicles can be seen. However as was noted earlier, some follicle remnants can be seen at higher magnification, and a ring of follicular epithelial cells is clearly seen amongst the lymphocytic/fibroblastic mass in Figure 3.17.d.

3.6.3. Discussion

These results suggest that the Leydig cells are important for thyroid allograft survival in the rat testis. Whether these cells perform specific immunosuppressive functions, or maintain immunosuppression by the actions of their steroid secretions remains to be elucidated. Important interactions have been demonstrated between Leydig cells and macrophages, and the successful destruction of macrophages by silica would have been a useful experiment. However, the actions of silica treatment appear very localised. That graft survival was not affected by this treatment cannot be taken to mean that macrophages are not implicated in the provision of an immunologically privileged environment in the testis. Indeed, in the silica-treated testes most of the interstitial region appeared quite normal and the macrophages (and Leydig cells) in these unaffected areas may have been responsible for graft survival. When Silica was

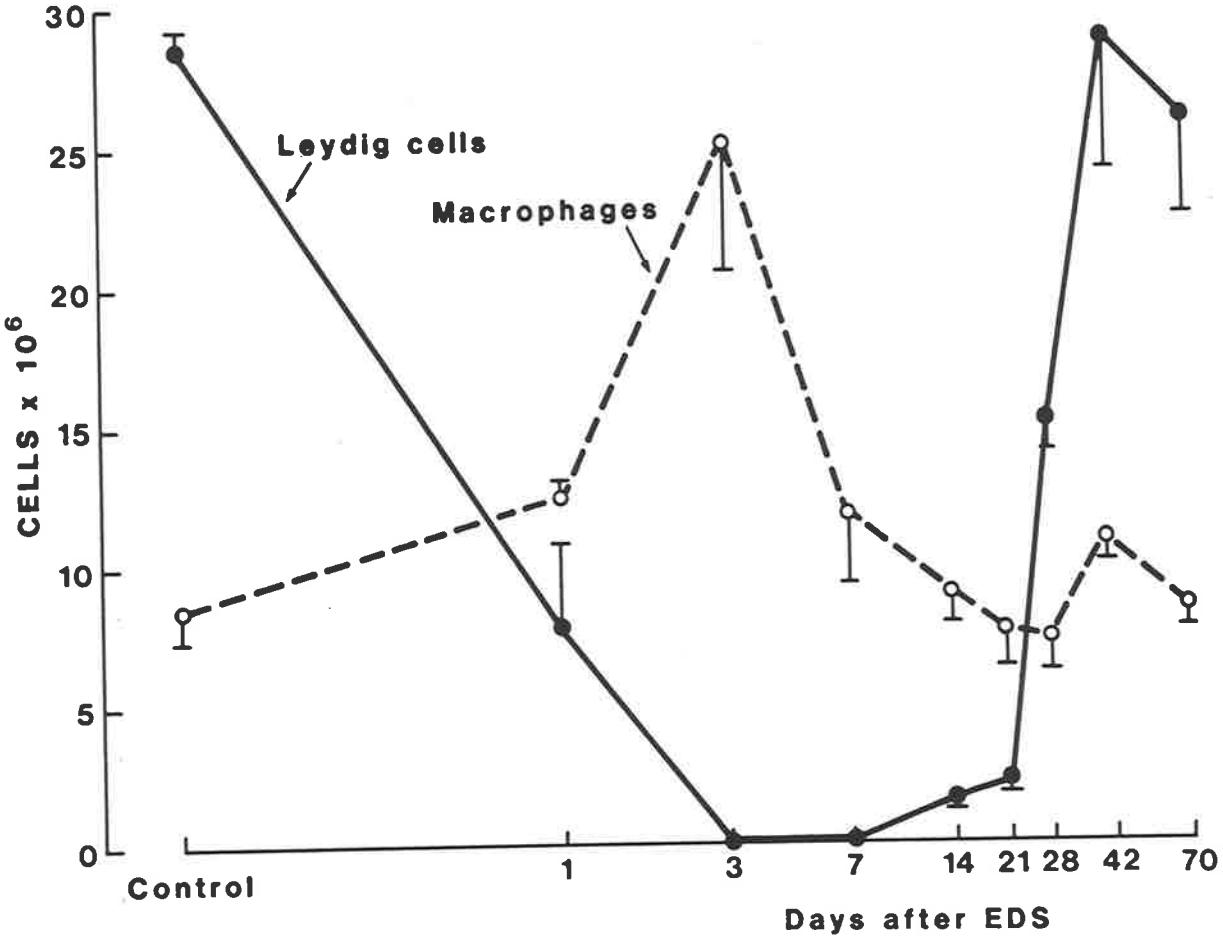
Figure 3.17. Sections from the testis of an adult rat 3 weeks after transplantation of an intratesticular thyroid allograft, an intratesticular injection of Silica, and treatment with EDS. (a) As noted previously, the region of Silica-induced damage is very localised. X 80. (b) The lack of Leydig cells in the interstitial region, and the atypical appearance of this tissue after EDS treatment is clearly evident, although there appears to be a more generalised presence of lymphocytes in the interstitial region than seen when EDS, or Silica is used alone. X 320. (c) The thyroid allograft has been rejected, and infiltrated by lymphocytes. X 80. (d) As noted with EDS treatment alone (see Figure 3.15.), some follicle remnants can be seen in graft zone (F). X 320.


a
b
c
F
d

used in conjunction with EDS, the reduced survival of thyroid allografts appeared to be due to the actions of EDS rather than silica. More detailed investigations of the actions of Silica on macrophages are obviously required before their involvement can be discounted, and unfortunately further investigations were not possible in this study.

The effects of EDS treatment on the immune privileged status of the rodent testis require further examination. While the rejection of allografts after such treatment may relate to the effects of EDS on Leydig cells, the recent findings of Kerr et al (1987) on macrophage numbers in the testis after EDS treatment requires discussion. As seen in Figure 3.18., macrophage numbers in the testis are significantly increased 3 days after EDS treatment. Most Leydig cells are also completely destroyed by this stage. While this increase in macrophages might be due to proliferation of testicular macrophages, the rapidity of the increase in numbers suggests that they may be circulating macrophages of extratesticular origin that infiltrate the testis to phagocytose the degenerating Leydig cells. This idea is supported by the apparent decline in macrophage numbers by 7-10 days after EDS administration. Indeed, Kerr et al (1985) report the destruction of Leydig cells with EDS to elicit an immune response involving macrophage-lymphocyte interactions, with large numbers of lymphocytes infiltrating the interstitial tissue 7 days after EDS treatment. The destruction of Leydig cells by EDS may affect the function of testicular macrophages, and cause or allow accumulation of extra-testicular marcrophages in the interstitial region. This might then allow normal processing of graft antigen to the host immune system as circulating macrophages are processed in lymph nodes,

Figure 3.18. Numbers of Leydig cells and Macrophages in the testes of adult rats up to 70 days after EDS administration (from Kerr et al, 1987).


30
25
20
15
10
5
0
CELLS x 10^6
Leydig cells
Macrophages
Control
1
3
7
14
21
28
42
70
Days after EDS

generating production of sensitized cytotoxic lymphocytes, and ultimate graft rejection. This is a very exciting hypothesis, and the importance of Leydig cells and macrophages for the immunologically privileged status of the rat testis warrants further investigation.

CHAPTER 4: THE OVINE TESTIS AS AN IMMUNOLOGICALLY PRIVILEGED SITE

4.1. INTRODUCTION

All previous work published on graft survival in the testis has used only rodents and related species as the recipients (see chapter 1). To my knowledge there is no description of the ability of the testis in other mammals to support allografts. As highlighted in the introductory review to this thesis, the anatomy of the interstitial region of the rodent testis is very different to that of other mammals. To use tissue grafts in the testis of animals for various studies of endocrine function and possibly growth manipulation would require them to have immunologically privileged testes. Likewise, the medical implications of much of this research would require the human testis to be an immunologically privileged site.

The anatomy of the interstitial region in the ovine testis is much more representative of that found in the larger mammals. With sheep readily available as a resource, it was obviously useful to examine the immune status of the ovine testis. Likewise, it was also of interest to examine testicular lymph for any hormones that might be secreted by tissue grafts in the testis, given that there was no indication in the earlier experiments that such hormones were leaving the testis in venous blood. Such studies were hampered in the earlier studies because of the inability to access the testicular lymphatics in the rat, due to their small size. However, a number of authors have described the cannulation of the testicular lymphatics in the ram (Lindner, 1963, 1967, 1969; Cowie et al, 1964; Morris and McIntosh, 1971; Ball and Setchell, 1983). Morris and co-workers (Hall and Morris, 1962, 1963, 1965; Hall et al, 1967; Lascelles and Morris, 1961; Pederson and Morris, 1970) have also used the sheep extensively

in immunological studies, and it was felt that this species would be most suitable for the studies required. While testicular lymph cannot be assumed to be exactly the same as the fluid in the interstitial region of the testis, it should provide an indication of changes occurring in the interstitium that might be important during allograft survival or rejection. This would be useful in following events after initiating various experimental manipulations of the interstitial environment. However before one could pursue the involvement of the lymphatics in transporting graft-derived hormones out of the testis, and the importance of lymphoid and interstitial fluid elements in providing immune protectection in the testis, it was necessary to demonstrate allograft survival in the ovine testis.

This chapter discusses the survival of autografted and allografted thyroid tissue, and allografted pituitary tissue in the ovine testis.

4.2. EXPERIMENTAL PROCEDURE

4.2.1. Animals

Adult Merino rams were obtained from the Institute flock and housed indoors in individual pens. Water was available ad libitum and animals were fed lucerne hay and sheep pellets (Milling Industries, Adelaide) once per day. All experiments were performed during the months of April and May towards the end of the breeding season but when animals were still sexually active.

4.2.2. Thyroid Transplants

Twelve rams were totally thyroidectomised as described in 2.3.1., and the recovered thyroid lobes were placed on ice to be used as graft material. Transplantation always occurred within ten minutes after thyroid removal. Six animals received intratesticular autografts and six received intratesticular allografts of one thyroid lobe into one testis, at the time of thyroidectomy as described in 2.3.8. The contralateral, ungrafted testis served as a control in each animal.

Three weeks after tranplantation animals were given a TRH challenge as described in 2.5.2.

Four weeks after transplantation, all animals received an intravenous bolus of 1 mCi Iodine-131 (see 2.5.1.2.) and were castrated 48 hours later under halothane (Fluothane, ICI Australia) anaesthesia. Thyroid hormones are largely transported in the circulation in association with serum binding proteins such as albumin (Robbins et al,1978) and Galil et al (1981) have shown that over 95% of albumin injected into the ram testis is cleared in lymph. Because of this, testicular lymph was thought possibly to be an important avenue for the transport to the general circulation of hormones produced by the grafts in the testis. To allow blood and lymph samples to be collected, polyvinylchloride (PVC) or polyethylene (PE) catheters were inserted into a testicular vein (2.3.5.2.) and a testicular lymphatic vessel (2.3.6.) in the spermatic cord of both testes prior to castration. Jugular venous blood was also sampled via an indwelling catheter (see 2.3.5.2.). Blood plasma and lymph samples were stored at -20^{o}C until assayed. After castration the distribution of I-131 was assessed in each testis as described in 2.5.1.2. Tissue

from the transplant site of the grafted testis was then processed for histological examination as described in 2.14. Paraffin sections were stained with haemotoxylin and eosin and examined by light microscopy.

Blood samples were taken at weekly intervals from these twelve animals and four other control rams of the same age and from the same flock, for hormone analysis. Sampling was begun six weeks prior to transplantation, and continued through the experiment until four weeks after castration.

4.2.3. Pituitary Transplants

In a second experiment, another four rams received intratesticular pituitary allografts. Four healthy Merino males castrated soon after birth (wethers) were selected as pituitary donors. In these animals, the lack of steroid feedback on the pituitary should have rendered the pituitaries hyperactive in terms of gonadotrophin secretion. The protocol adopted in this experiment was initially to prepare the testis of the recipient animal in the same way as used for thyroid transplants (see 2.3.8.). Donor pituitaries were obtained just prior to transplantation as described in 2.3.2. and placed on ice until transplanted as described in 2.3.8., and no more than ten minutes elapsed between the removal of the donor's head and the transplantaion of the isolated pituitary into the recipient testis.

To ensure that the pituitaries were functioning normally prior to transplantation, donor wethers were challenged with an intravenous bolus of Luteinising Hormone - Releasing Hormone (LHRH) three weeks prior to surgery as described in 2.6. At the same time, the rams that

were to be used as graft recipients were infused with LHRH as described in 2.6.2.1. to check that a concentration of LHRH which, if infused into the testicular artery to provide sufficient local concentrations of LHRH to stimulate gonadotrophin secretion by viable transplants (see 2.6.2.2.), would not be sufficient in the peripheral circulation to stimulate the animal's own pituitary into secreting gonadotrophins.

Four weeks after transplantation all animals were castrated. To examine graft function before the testis was removed, an expanded catheter was inserted into the testicular artery on the surface of the testis and LHRH was infused through this catheter (see 2.6.2.3.) for one hour to stimulate gonadotrophin secretion by the grafts. Viable pituitary grafts should secrete protein hormones under such a stimulus, and these would probably leave the testis in testicular lymph and venous blood. Catheters were therefore placed into a testicular vein and lymphatic as described in 2.3.5.2 and 2.3.6. Sampling was begun 20 min prior to LHRH infusion and continued for two hours afterwards at 10 minute intervals.

After castration, tissue from the graft site and a comparable piece of tissue from the control testis were placed in Picric Acid/Formaldehyde fixative and processed for immunohistochemistry as described in 2.15.

Blood samples were taken weekly from these four animals, and throughout the experiment from four other control rams of the same age and from the same flock, to monitor gonadotrophin levels.

4.2.4. Immunohistochemistry

Cryostat sections of graft tissue and normal pituitary were prepared and processed for immunohistochemical localisation of Follicle-stimulating hormone (FSH), Luteinising hormone (LH), Prolactin (Prl) and β-Endorphin (End) as described in 2.15.2. Sections were mounted in buffered glycerol and examined by fluorescent microscopy.

4.2.5. Hormone Assays

Plasma and lymph concentrations of LH, FSH, and TSH were measured using double-antibody radioimmunoassay procedures as described in 2.13.3.2, 2.13.3.4 and 2.13.3.7. Concentrations of these hormones are expressed relative to the following standards : LH (NIADDK-oLH-24), FSH (NIADDK-oFSH-15), TSH (NIADDK-bTSH-I-1). A direct radioimmunoassay was used to measure serum T_4 as detailed in 2.13.2.

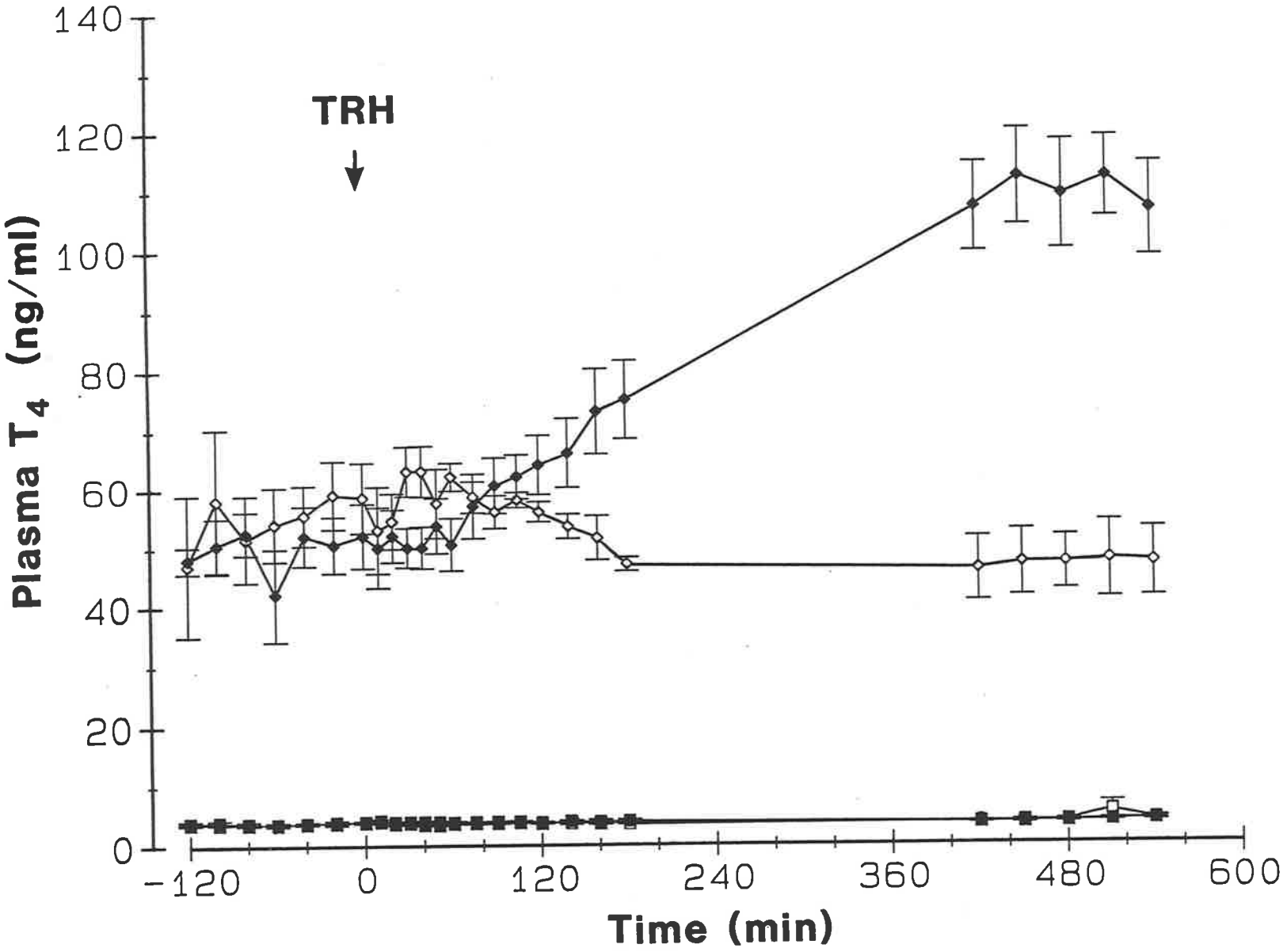
4.3. RESULTS

4.3.1. Thyroid Transplants

4.3.1.1. TRH challenge

The TRH challenge 3 weeks after transplantation stimulated T_4 secretion in intact animals as seen in Figure 4.1., but did not have any effect in the thyroidectomised thyroid-transplanted animals, whether the grafts were auto- or allografts.

Figure 4.1. Plasma T_4 levels in Control (solid and open diamonds), Thyroidectomised-thyroid autografted (open squares) and Thyroidectomised-thyroid allografted (solid squares) Merino rams, following intravenous injection of either 2ml physiological saline (one group of Control animals only, open diamonds) or 200 ug TRH in 2ml physiological saline (second group of Control animals, solid diamonds, and all transplanted animals, solid and open squares). Values plotted are means (n=6) $\pm$ standard error of the mean.


TRH
Plasma T_4 (ng/ml)
140
120
100
80
60
40
20
0
−120
0
120
240
360
480
600
Time (min)

4.3.1.2. Iodine localisation

Typical photographs obtained with computerised gamma photography are shown in Figure 4.2. While five of the six autografted testes showed specific iodine localisation in the region of the graft (Figure 4.2.a), none of the six allografted testes showed any such localisation (Figure 4.2.b, $P < 0.025$ by X^2 test). A cross-sectional scan of tissue counts through the testis shows a significant concentration of counts at the autograft site when compared to background (Figure 4.2.c) while the allograft counts could not be distinguished from background levels (Figure 4.2.d).

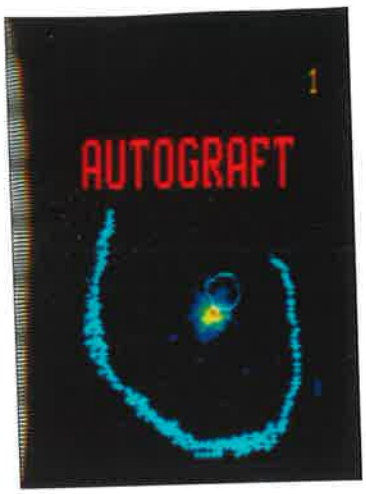
4.3.1.3. Histology

Figure 4.3 shows representative sections of the grafts at four weeks after transplantation. While groups of clearly defined, healthy thyroid follicles containing colloid can be seen in the autograft (Figure 4.3.a) no such tissue was found in the allograft, although follicle remnants could be seen (Figure 4.3.b).

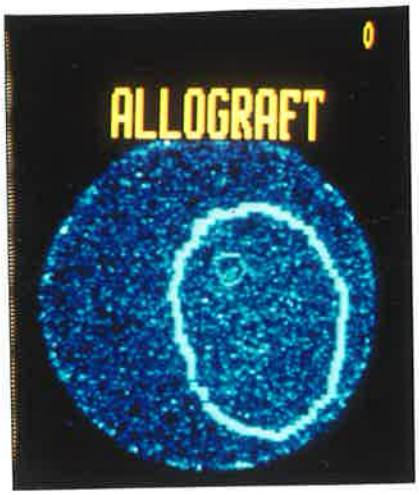
4.3.1.4. Plasma and lymph hormone concentrations obtained at castration

No significant levels of thyroxine could be detected in the venous blood or lymph obtained from any of the testes at the time of surgery (see Figure 4.4.).

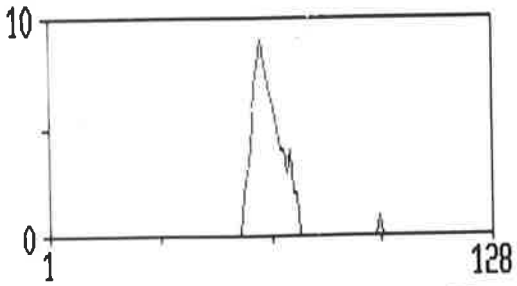
Figure 4.2. Representative computerised gamma photographs of iodine accumulation in testes of thyroidectomised Merino rams containing thyroid autografts (a) or thyroid allografts (b), 48 hours after administration of 1 mCi ^{131}Iodine. The outer heavy line shows the outline of the testis and the smaller open circle the site of the suture in the tunica. In the cross-sectional scans of counts for both grafts (c,d), ordinate values indicate units of field width, and abcissa values indicate ratio of counts relative to background. Abcissa values in (c) are not directly comparable to those in (d), where background values have not been subtracted.



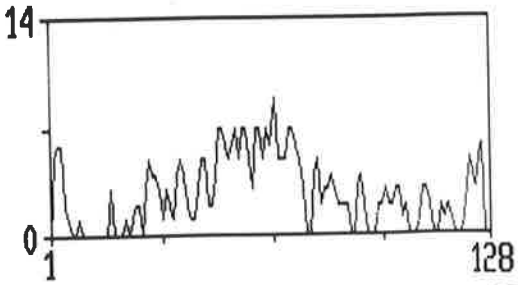
a



b



c



d

Figure 4.3. Representative histological preparations of intratesticular thyroid autografts (a) and thyroid allografts (b) four weeks after transplantation. X 320.

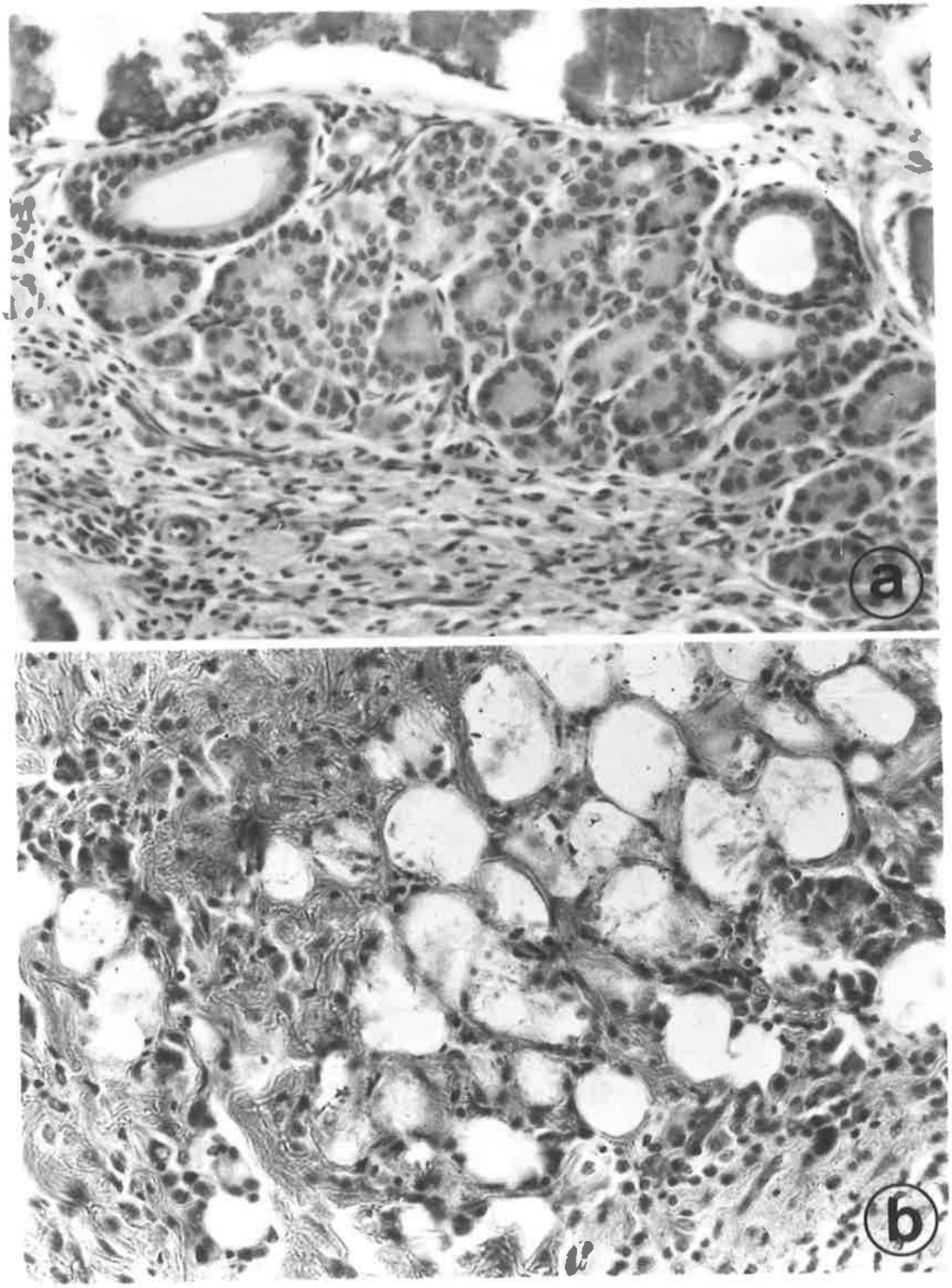
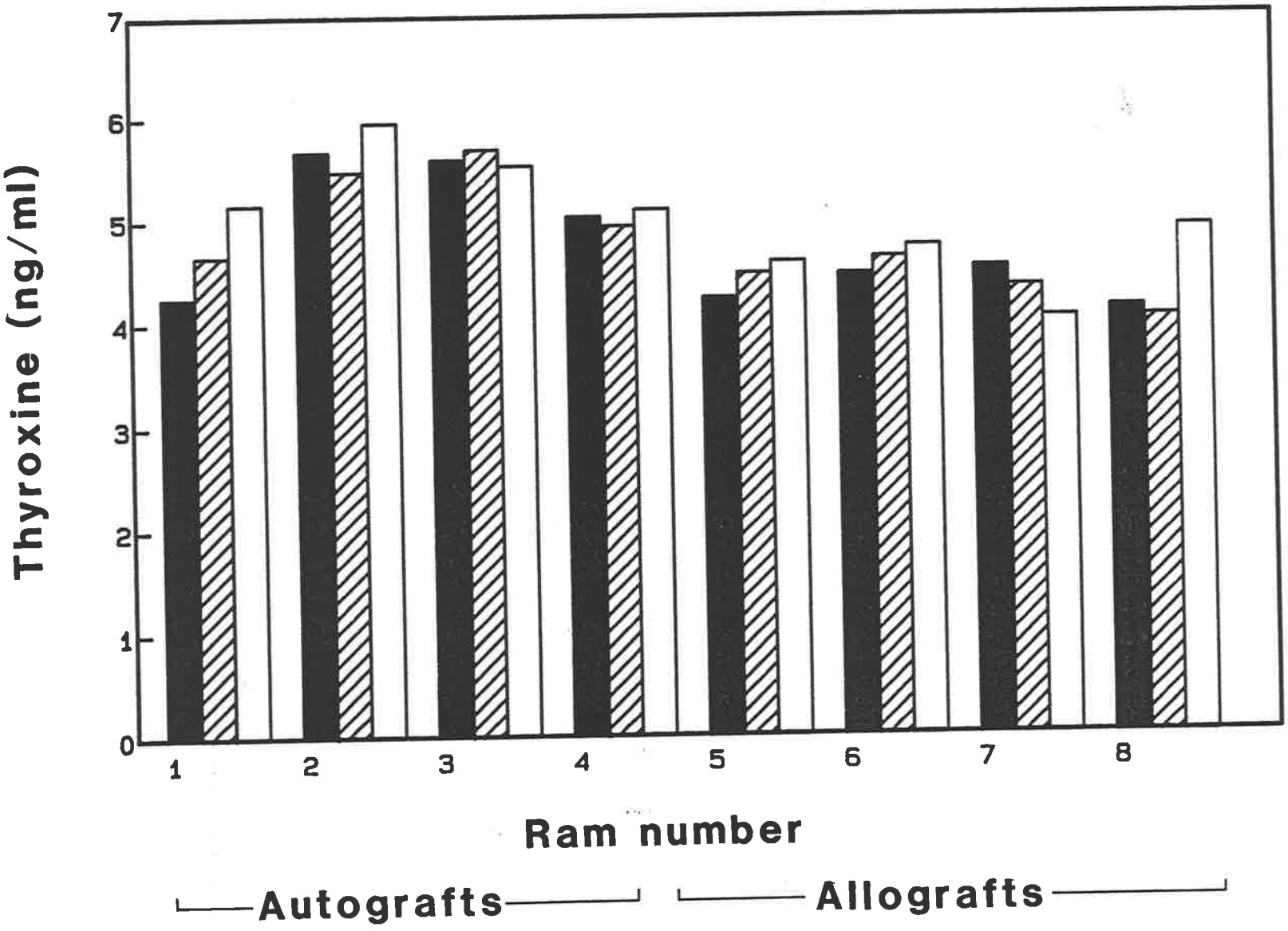

a
b

Figure 4.4. Plasma levels of T_4 in peripheral blood (solid bars) and testicular venous blood from transplanted (open bars) and control (hatched bars) testes, three weeks after thyroid transplantation. Values plotted are individual levels for Autografted (numbers 1-4) and Allografted (numbers 5-8) rams.


Thyroxine (ng/ml)
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
Ram number
Autografts
Allografts

4.3.1.5. Plasma hormone concentrations in weekly samples

Levels of plasma T_4 and TSH measured in weekly blood samples from all animals are shown in Figure 4.5. Control animals showed a reasonably stable plasma T_4 level while associated TSH levels were basal (Figure 4.5.a). There was no difference in the hormone profiles of autografted (Figure 4.5.b) or allografted (Figure 4.5.c) animals and in all transplanted animals, T_4 levels fell to baseline values within two weeks of thyroidectomy. TSH levels rose as a consequence, peaking 4-6 weeks after thyroidectomy.

4.3.2. Pituitary Transplants

4.3.2.1. LHRH challenge

Figure 4.6. shows the response of donor wethers and recipient rams to the pre-transplant LHRH challenge. All wethers showed a significant LH secretion in response to the injected LHRH (single dose of 5 ng/kg bw). The one-hour infusion of LHRH (total dose of 2 ng/kg) into the lateral saphenous vein of recipient rams did not stimulate LH secretion significantly.

4.3.2.2. Plasma and lymph hormone concentrations

The infusion of LHRH into the testicular artery failed to induce any secretion of LH or FSH in any of the animals. Unstimulated levels in venous blood and lymph from grafted testes were no different to those from samples from the control testes or peripheral blood.

Figure 4.5. Plasma levels (ng/ml) of T_4 (solid squares) and TSH (open squares) in weekly blood samples collected from (a) control, (b) thyroidectomised-thyroid autografted and (c) thyroidectomised-thyroid allografted Merino rams. Samples were not taken in weeks 9 or 11. In treated animals, T indicates the time of thyroidectomy and transplantation, and C indicates the time of castration. Values plotted are means (n=6) $\pm$ standard error of the mean.

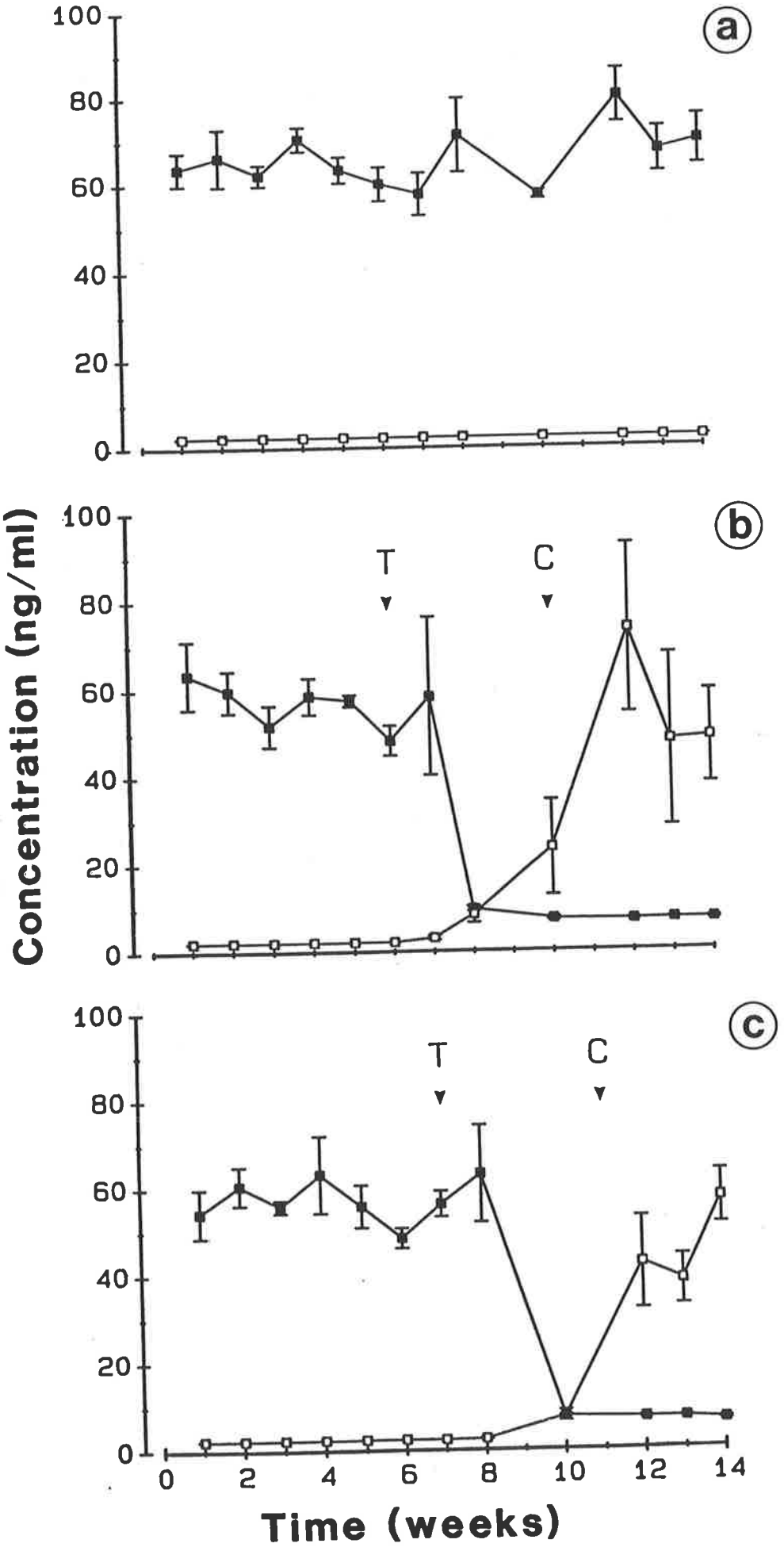
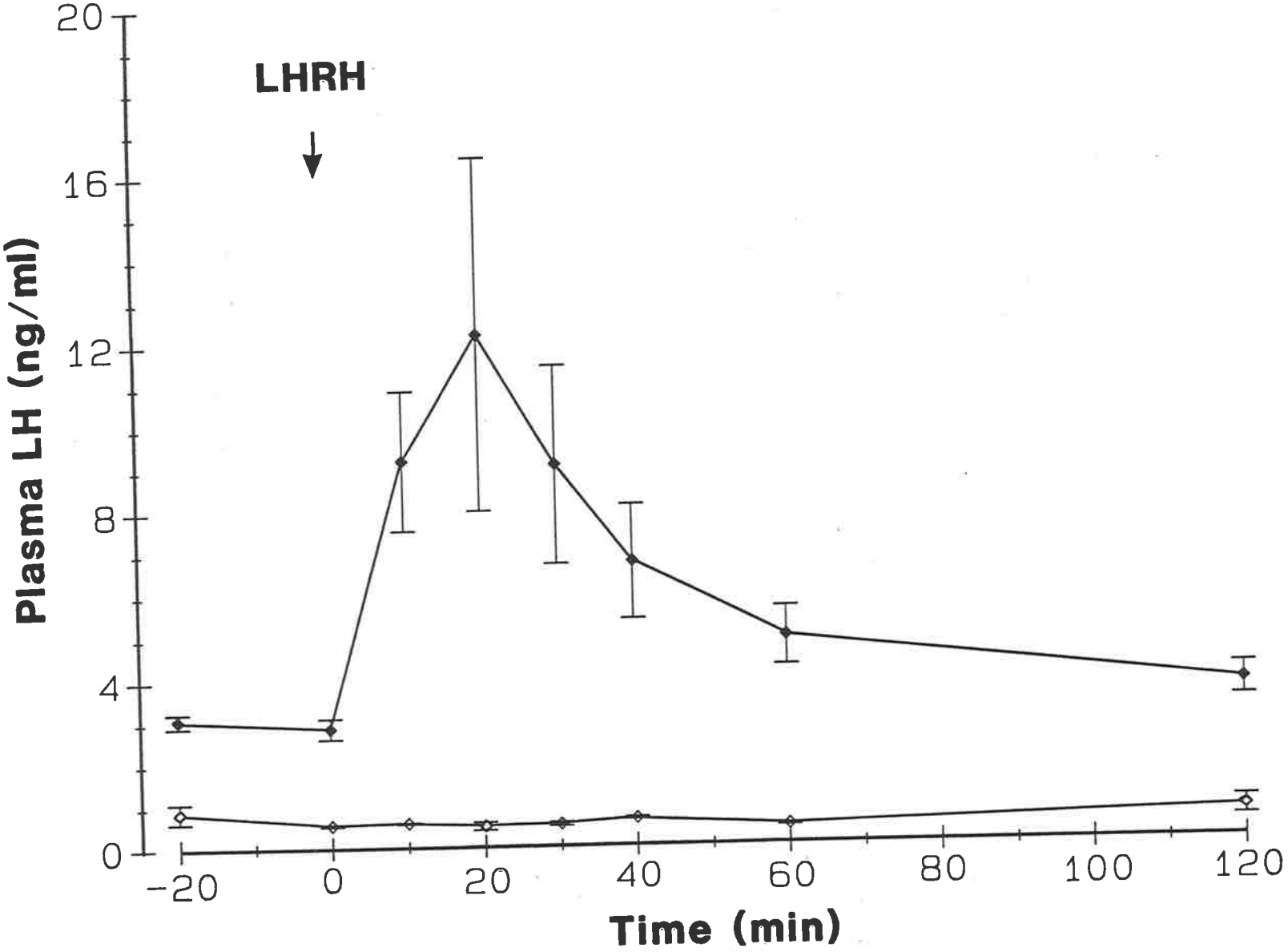

a
b
c
T
C
T
C
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Concentration (ng/ml)
Time (weeks)

Figure 4.6. Levels of LH (ng/ml) in jugular plasma of castrated (Wethers, solid diamonds) and entire (Rams, open diamonds) male Merino sheep following intravenous administration of LHRH. Wethers received a single bolus injection of 5ng LHRH/ kg bw into the jugular vein, and rams received an intravenous infusion of 2ng LHRH/min for 1 hour, into the lateral saphenous vein (see text for details). Time of injection or beginning of infusion is indicated. Values plotted are means (n=4) $\pm$ standard error of the mean.


LHRH
Plasma LH (ng/ml)
20
16
12
8
4
0
-20
0
20
40
60
80
100
120
Time (min)

4.3.2.3. Immunohistochemistry

Fluorescent-conjugated antibodies failed to reveal any significant regions of surviving tissue in any of the grafts. Control pituitary tissue however was well stained as seen in Figure 4.7., and showed clearly defined cells specifically stained for the given hormone tested. While the graft regions showed some staining, this was largely non-specific and any specifically stained cells appeared to be invasive phagocytes.

4.3.2.4. Plasma hormone concentrations in weekly samples

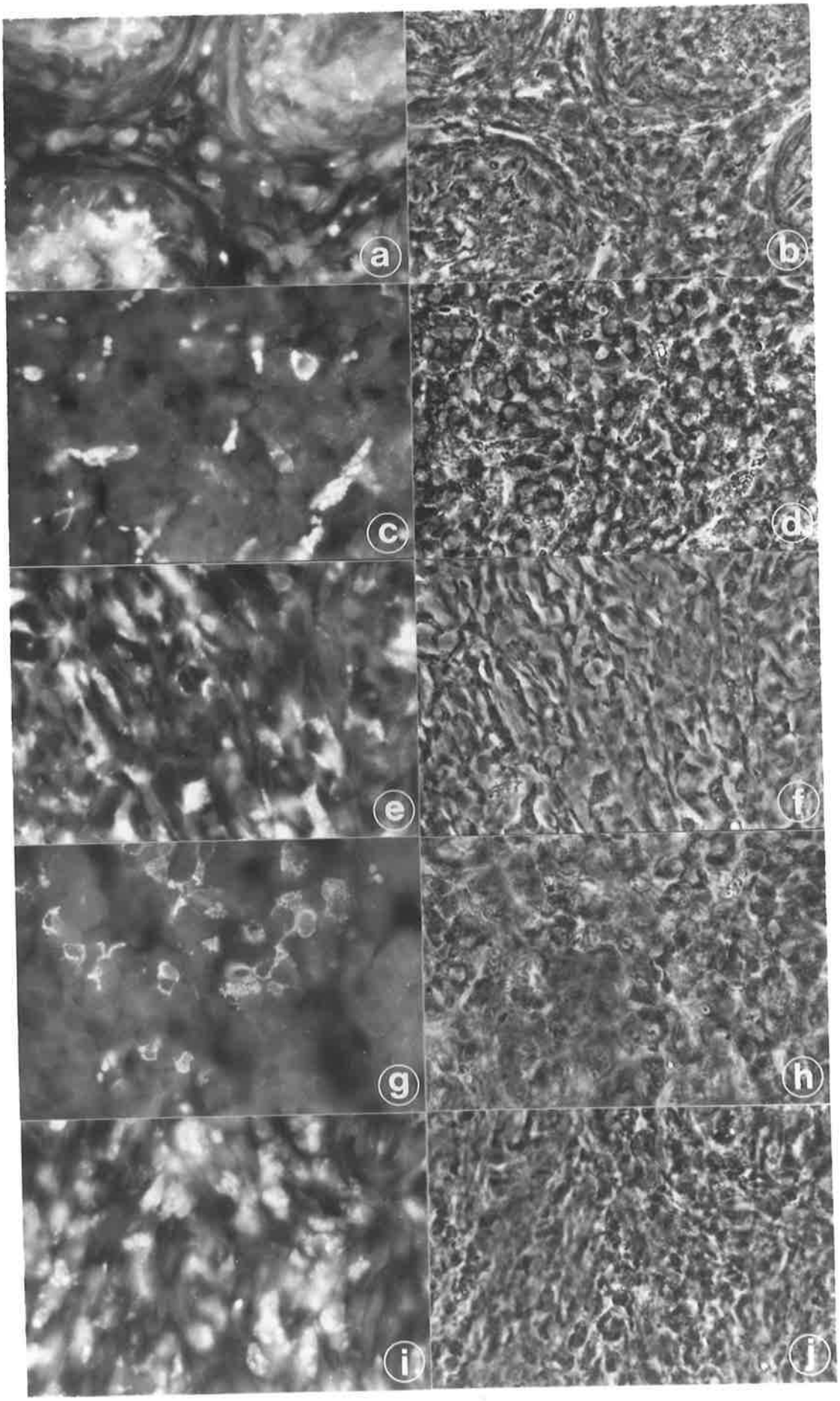
Pituitary grafts had no impact on circulating LH or FSH levels in any of the animals.

4.4. DISCUSSION

The results of these experiments clearly demonstrate rejection of endocrine allografts by the ovine testis. This is in direct contrast to similar experiments performed in rodents (Dib-Kuri et al, 1975; Head et al, 1983a; Whitmore and Gittes, 1979). It appears that the immune competance of the ovine testis is superior to that of rodents and it is not correct to make the generalization that the mammalian testis is an immunologically privileged site.

Because thyroid autografts were not rejected, as judged by iodine accumulation and histology, the techniques adopted for transplantation would appear to be satisfactory although it was surprising that the

Figure 4.7. Immunohistochemistry of representative sections of control Testis (a,b), control Pituitary (c,d) and intratesticular pituitary Allograft (e,f) treated with fluorescent-conjugated anti-ovine FSH; and control Pituitary (g,h) and intratesticular pituitary Allograft (i,j) treated with fluorescent-conjugated anti-human End. Each fluorescent image (a,c,e,g,i) is accompanied by its phase-contrast image (b,d,f,h,j). X 180.


a
b
c
d
e
f
g
h
i
j

thyroid hormone profiles of autografted and allografted animals were not different. However, a number of explanations may account for this. The time between transplantation and graft removal may have been too short to allow sufficient recovery of graft tissue for T_4 secretion. It is more likely that the surviving pockets of thyroid tissue in the autografts may have secreted T_4, but the small amount of tissue meant that levels produced were below the sensitivity of the assay.

CHAPTER 5. THE IMMUNOLOGICALLY PRIVILEGED TESTIS - GENERAL DISCUSSION

5.1. DISCUSSION

The processes involved in initiating and effecting the rejection response are still not completely understood. While efficient lymphatic drainage is necessary for prompt rejection of some grafts (Barker and Billingham, 1968), there is now considerable evidence that the critical events in the rejection response occur in the graft itself (Pederson and Morris, 1970; Ascher et al, 1981). A number of cell types have been shown to participate in allograft rejection (Hayry et al, 1984) including T-cells, Natural Killer cells and Macrophages. However the authors of such reports have also indicated that rejection ultimately depends on the development of a generalised inflammatory response, initiated and regulated by specific antigen-related lymphocytes. There are therefore numerous processes occurring locally in the graft that might be subject to interference from the environment of the graft and previous workers have demonstrated that the immunologically privileged status of the rodent testis is probably due to some factor or factors within the testis itself. Testicular-derived factors that may contribute to such interference and account for this privileged status have recently been reviewed by Head et al (1983a, 1983c) and Head and Billingham (1985). Suggestions include the Leydig cells because of their ability to adhere spontaneously to lymphocytes and to suppress non-specific proliferation of mitogen-stimulated and alloantigen-stimulated lymphocytes in vitro (Born and Wekerle, 1982); the germ cell population because of their ability to impair T-cell responses to lymphocytes, and lymphocyte responses to mitogens (Shearer and Hurtenbach, 1982; Hurtenbach and Shearer, 1982); and various products

such as androgens, progesterone and prostaglandins which can be shown to be immunosuppressive in vitro (Clemens et al, 1979; Roubinian et al, 1977). Experiments altering endogenous steroid levels, or affecting various cells of the immune system have been employed in attempts to determine the mechanisms underlying testicular immune privilege (Stites and Siiteri, 1983; Hurtenbach and Shearer, 1982).

The experiments reported in chapter 3 have confirmed that the rodent testis does possess some degree of immune privilege. The results presented demonstrate that the seminiferous tubules are unlikely to be involved in protecting allografts, since impairment of their normal functions by cryptorchidism and efferent duct ligation has not adversely affected graft survival. The destruction of germ cells by experimental cryptorchidism has not affected graft survival, and they are thus unlikely to be involved in conferring immune protection on allografts in the interstitium. Likewise, the various effects on Sertoli cells caused by these two treatments suggest that they are unlikely to be the prime instigators of an immunologically privileged environment in the interstitial tissue, although their precise role in contributing to the privilege afforded this site cannot be altogether discounted. Whitmore et al (1985) concluded that Sertoli cells were responsible for the immunologically privileged status of the testis, after studies in rats with Sertoli-cell only testes created by in-utero irradiation. This treatment results in sterile testes, but does not adversely affect the interstitial cells (Means and Huckins, 1974), and therefore the conclusions of these authors about the importance of the Sertoli cell is probably incorrectly founded. In the present study, the slower lymph flow from

the rat testis has also been found to be of little importance in protecting allografts in the testis from immunological rejection.

The conclusion drawn from these initial investigations is that the factors responsible for the immune privilege afforded the rat testis are probably located within the interstitial region of the testis. As indicated, the Leydig cells and their androgenic products are possibly implicated. Graft survival in rats was greatly affected by the destruction of Leydig cells with EDS which may implicate the Leydig cells and/or their products in protecting allografts from immune rejection in the testis. However, the accumulation of macrophages and lymphocytes in the interstitial tissue after this treatment (Kerr et al, 1985) suggests that the immune system may react to EDS treatment, and that grafts are rejected as a consequence of this treatment, rather than as a result of the loss of Leydig cell function. Apart from the Leydig cells, other cell types known to occur in the testicular interstitium may also be involved, and the mast cells, macrophages, and mesenchymal cells found in this region must also be considered. Preliminary experiments described in chapter 3 attempted to examine the involvement of testicular-macrophages using silica to destroy these resident macrophages as described by Hovatta et al (1986). However, this treatment was found to be very localised in its action - a finding not reported by Hovatta et al (1986) (who in fact make no mention of the histology of the testis after such treatment). The importance of testicular macrophages for allograft survival in the testis remains to be determined.

The results presented in chapter 4 indicate that the ovine testis is not an immunologically privileged site. The mammalian testis can no

longer be generally referred to as immunologically privileged since one exception has now been demonstrated, and from an anatomical perspective, the privileged testis may in fact be a rodent-specific phenomenon. The discrepancy in immune status of the ovine and rodent testis is unlikely to involve steroids as the sole factor in immunosuppression. Firstly, the types of steroids present, and their relative concentrations, would have to be appreciably different between sheep and rats to account for the discrepancies in allograft survival reported in the previous two chapters. However, such data as is available suggests that the steroid profile in these two species is not greatly different (see Setchell, 1978). Secondly, the importance of steroid involvement remains questionable because of the unphysiologically "high" concentrations needed in-vitro to produce immunosuppression (Wyle and Kent, 1977; Holdstock et al, 1982). It is interesting that Head and Billingham (1985) have reported the reduced survival of intratesticular parathyroid allografts in rats pretreated with a single injection of oestrogen. These animals had low circulating levels of testosterone although intratesticular and testicular venous levels of testosterone do not appear to have been examined. The Leydig cell is the likely source of oestrogen in the adult rat testis (Payne and Valladares, 1980) and Mulder et al (1974) have demonstrated oestradiol receptors on Leydig cells. Low peripheral levels of testosterone after oestrogen treatment have been shown by other authors to be due to direct inhibition of Leydig cell function (Kalla et al, 1980; Tsai-Morris et al, 1985). Head and Billingham (1985) proposed that locally produced hormones might be the key to testicular immune privilege. They suggested that the reduced survival of intratesticular parathyroid allografts in rats injected 1-2 days

prior to grafting with oestradiol cypionate was due to a reduced level of immunosuppression in the testis due to the lower intratesticular levels of androgen. However, exogenous oestrogen treatment also suppresses pituitary LH secretion (Gay and Dever, 1971; de Jong et al, 1975), and might therefore affect other cells in the testis and body generally. Kalla et al (1980) have also reported testicular levels of testosterone to be reduced after oestradiol treatment, but found pregnenolone and in particular progesterone levels to increase significantly. Pregnenolone is more immunosuppressive in vitro than testosterone (Wyle and Kent, 1977; Pavia et al, 1979) which suggests that the results of Head and Billingham (1985) might not be simply due to a lack of steroid inhibition of the immune response. In 1969, Vernon-Roberts reviewed the effects of steroid hormones on macrophage activity. Oestrogens have a strong stimulating effect on the macrophage system generally, and oestrogen injection results in the appearance of large numbers of macrophages in the spleen and liver and the mobilization of these cells into the circulation (Helmy and Nicol, 1951; Nicol and Helmy, 1951; Nicol et al, 1952). Following a single subcutaneous injection of 17β-estradiol in the mouse, Vernon-Roberts (1969) observed two peaks of increased numbers of lymphocytes in the peritoneal cavity on the first and sixth days after injection. Both peaks of increased lymphocytes were followed 24 hours later by an increase in the number of cells intermediate in appearance between lymphocyte and macrophage, and 48 hours later by an increase in the number of mature macrophages. Head and Billingham (1985) would therefore have made their grafts at a time of increased immune activity. Leydig cell function was also affected at this time and testosterone levels were significantly reduced, which may have

affected other cell types in the testis and reduced the immunosuppression normally found in this site. These reports indicate that the effects of an oestrogen injection are not confined to the Leydig cell, and it is therefore unwise to assume that the reduced survival of parathyroid allografts in the testis after oestrogen treatment was only due to an impairment of Leydig cell function. Other factors besides steroids are likely involved in immune suppression in the testis.

There remain therfore, two fundamental areas for examination and comparison between the two species used in the present experiments (if not in other species, as well). Firstly, we need to be able to compare the interstitial environments of the ovine and rodent testis, examining the various physiological aspects of their function, steroid production, and types and levels of steroids and other factors produced; and secondly to examine the various immunocompetant cells in the interstitial region of the testis, and to understand their functions, and relationships with other cell types of this region.

CHAPTER 6: MATERIALS AND METHODS

In addition to the materials and methods outlined in chapter 2, the following materials and methods were used in the studies to be presented in this section.

6.1. REAGENTS

Testosterone, Pregnenolone, hCG, and Coomassie Brilliant Blue G-250 were obtained from Sigma Chemical Co., St. Louis, Missouri,USA. Radiolabelled ^{3}H-Testosterone, ^{22}Na-Sodium Chloride, ^{14}C-Mannitol, ^{3}H-Water, and ^{125}I-Sodium Iodide were obtained from Amersham, Australia.

6.2. HEATING OF TESTES

Localised heating of the testes at 43 ^{o}C for 30 minutes, was achieved by anaesthetising the animals with sodium pentobarbitone (Nembutal, Abbotts; 60 mg/kg bw) and placing them on two sheets of perspex lying across a water bath that was maintained at 43 ^{o}C. The sheets of perspex were slightly separated so as to allow the animals scrota to hang down into the water which was kept at a level just below the perspex to ensure complete warming of the scrotum. Animals were left in this position for 30 minutes. They were then returned to their cages after recovery.

6.3. PREGNENOLONE

Pregnenolone was dissolved in Sesame oil, and administered to hypophysectomised animals with daily subcutaneous injections for

fourteen days. Animals were hypophysectomised as described in 2.3.2, and pregnenolone injections were commenced on the day of hypophysectomy.

6.4. TESTOSTERONE IMPLANTS

Lengths of medical-grade silastic tubing (4.7 mm O.D., 3.4 mm I.D., Dow Corning, Michigan, USA) were prepared prior to filling by closing one end with silastic adhesive (Silicone Type A adhesive, Dow Corning, Michigan, USA). A small funnel was used to pack testosterone (Sigma) into the tubing, and when well packed, the implant was sealed with another plug of silicone adhesive. Implants were stored in a dessicator at 4 ^{o}C until used.

Animals were anaesthetised with sodium pentobarbitone, and a small incision made in the skin over the abdomen. Using a blunt-ended probe, a subcutaneous pocket was created of sufficient size to contain the required implant. After feeding the implant into the pocket, the opening was sutured closed.

6.5. CELL DISPERSION TECHNIQUE

A fluid can be obtained from the testis after dispersion of testicular cells and centrifugation. The testicular capsule was carefully removed with as much of the testicular artery as possible. The rete testis is also removed with the capsule (Setchell, 1978). The resulting tubular mass was carefully transferred to a 5 ml disposable syringe to which was attatched a 21G needle. The tissue in the syringe was expelled through the needle into two Eppendorf tubes, and

immediately centrifuged at 8000 g for 5 minutes. The resulting supernatant was collected and stored at -20 ^{o}C until assayed.

6.6. HORMONE ASSAYS

6.6.1. Testosterone radioimmunoassay

Plasma testosterone concentrations were determined using antisera raised in a ewe against a testosterone-3-carboxymethyloxime-BSA conjugate (Dr. R.I. Cox, Hormone Assay Development Group, CSIRO Division of Animal Production, Blacktown, N.S.W.). The specificity of this antisera is shown in Table 6.1. The details of the procedures followed were as described by D'Occhio (1981). Briefly, duplicate aliquots of plasma (from 5 ul to 100 ul depending on testosterone concentration) were added to disposable glass culture tubes (12 x 75 mm) and the volume adjusted to 100 ul with gel PBS. After adding 1 ml of toluene : hexane (2:1) mixture, the plasma was vigorously extracted for 1 minute on a vortex. The plasma was then frozen in a liquid nitrogen-ethanol bath and the solvent extract was decanted in to another set of culture tubes. The solvent mix was then dried under a stream of nitrogen gas (40 ^{o}C). Standards (0 to 1600 Pg, testosterone in 100 ul gel PBS) were also extracted and dried the same way as the samples. These standards were mixed with (1, 2, 6, 7 (n)- ^{3}H) testosterone (approx. 20 x 10^{3} dpm in 200 ul gel PBS, Specific Activity 93 Ci/m mol) and antisera (in 200 ul gel PBS; 1:20,000 final dilution). Equivalent amounts of radiolabelled testosterone and antisera were also added to dried extracts of samples. Assay tubes were incubated at 4 ^{o}C for at least 12 h. Free and protein-bound

Table 6.1. Specificity of data for testosterone antisera

Steroid	% cross reaction* A	B
Testosterone	100.00**	100.00
5α-Dihydrotestosterone	31.00	35.00
4α-androstene-3β,17β-diol	30.00	
4α-androstene-17β,19-diol-3-one	3.5	
Androstenedione	1.3	2.0
Epitestosterone (17α-hydroxy-4-androstene-3-one)	0.11	
Etiocholanolone (5β-androstane-3α-ol-17-one)	0.10	
Androsterone (5α-androstane-3β-ol-17-one)	0.02	
Dehydroepiandrosterone (5-androstene-ol-17-one)	<0.01	
Oestradiol-17β	0.10	0.07
Oestrone	<0.003	
Oestriol	<0.003	
Progesterone	<0.004	
Cortisol	0.003	

* Calculated from the amount of steroid required to suppress maximum binding of ^{3}H-testosterone by 50%.

** Values for testosterone arbitrarily set at 100%. Cross reactivity determined by (A) Dr. M. Wong, Hormone Assay development Group, CSIRO, Division of Animal Production, (B) Dr. M.J. D'Occhio, Department of Animal Sciences, Waite Agricultural Research Institute. This table was adapted from M.J. D'Occhio (1981).

steroids were separated by the addition of 200 ul of PBS containing Dextran and Charcoal (62.5 mg Dextran, 625 mg Charcoal in 100 ml of PBS). The tubes were allowed to stand at 4 ^{o}C for 15 min and were then centrifuged at 800 x g for 20 min. The supernatant (bound fraction) was transferred to scintillation vials and radioactivity was determined using the scintillation system described in Section 2.13.1.4.

Sensitivity (2 x standard deviation of the buffer blank) was 5 pg and intraassay and interassay coefficients of variation (standard deviation/mean x 100%) were 6.0% and 8.9%, which were calculated from ten replicates of a sample run in the same assay and from three samples of low, medium and high concentration, run in duplicate, in each assay respectively.

Plasma testosterone concentrations were determined without chromatography after solvent extraction. However the only steroids which have been shown to cross react with the antisera used are 5 a-dihydrotestosterone and androstenediol (Table 6.1) (D'Occhio, 1981). Since the levels of these androgens in rams, boars and rats (the species of interest in the present studies) were very low relative to testosterone, they do not contribute significantly to the testosterone values (Falvo and Nalbandov, 1974; Tremblay et al, 1970; Schanbacher, 1976).

6.7. PROTEIN ASSAYS

Protein measurements were made using an adaption of the

Protein-Dye binding Microprotein Assay of Bradford (1976).

6.7.1. Protein Reagent

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. 100 ml of 85% (w/v) phosphoric acid was added, and the solution diluted to a final volume of 1 litre.

6.7.2. Protein Assay

Protein solution containing up to 1 mg/ml protein in a volume of 20 ul was dispensed into 1 ml cuvettes. 1 ml of the protein reagent (6.7.1.) was added to each cuvette. After mixing by inversion, absorbance at 595 nm was measured after 2 minutes and before 15 minutes, against a reagent blank prepared from 20 ul of the appropriate buffer and 1 ml of the protein reagent. Measurements were made using a Perkin-Elmer Lambda 5 UV/Visual Spectrophotometer. Standards of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml protein were prepared using bovine serum albumin. By plotting the weight of protein against the corresponding absorbance, a standard curve was generated that could be used to determine the protein in unknown samples. Such a curve is shown in Figure 6.1. Complex stability was reported by Bradford to be satisfactory for up to one hour after mixing with sample. All samples in this study were measured by 15 minutes after addition of protein reagent, and as seen in Figure 6.2., absorbance was quite stable during this period of time.

Figure 6.1. Standard curve for the measurement of protein using the microprotein method of Bradford (1976).

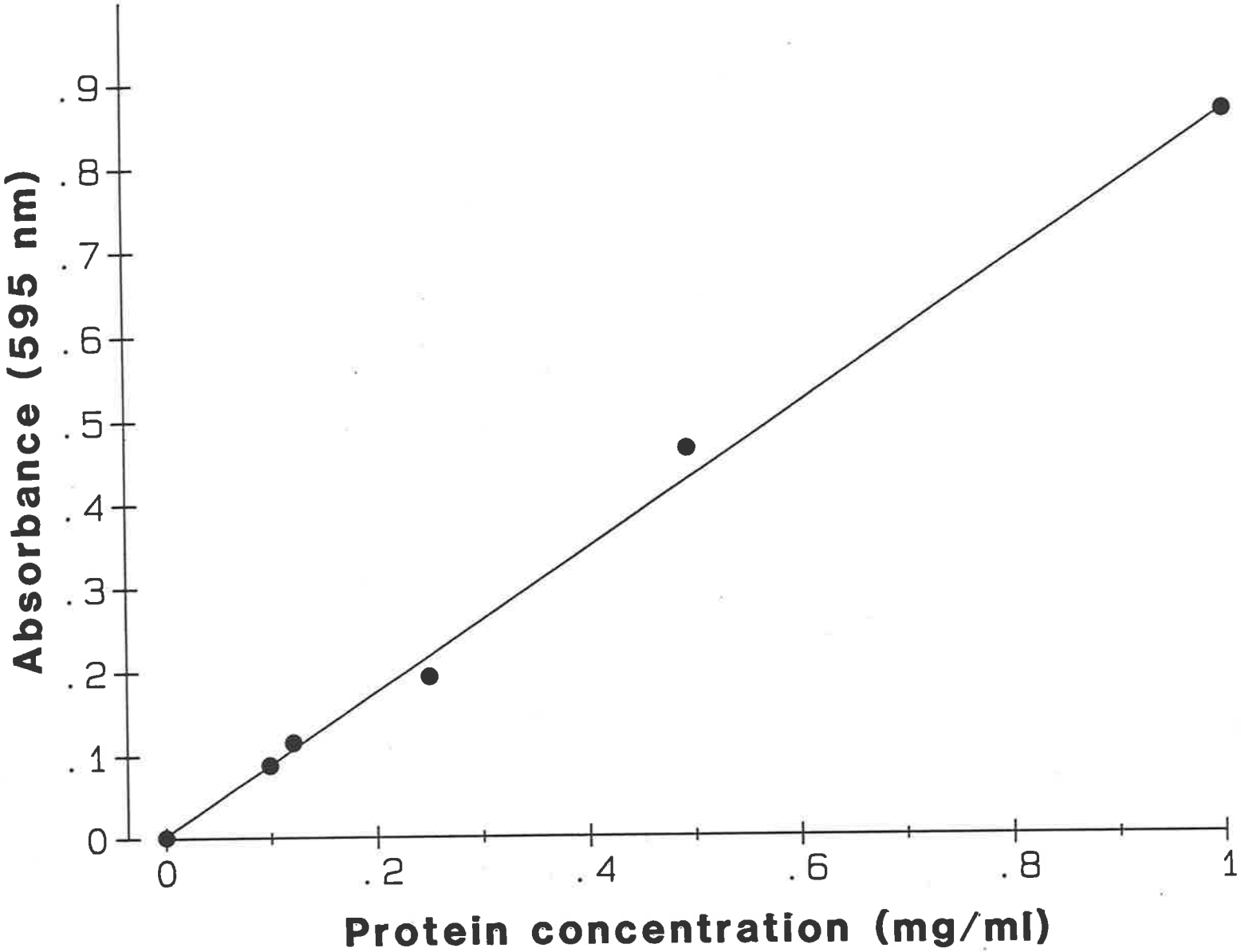

Absorbance (595 nm)
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0
.2
.4
.6
.8
1
Protein concentration (mg/ml)

Figure 6.2. Absorbance spectrum at 595 nm for dye-protein complex, indicating complex stability for more than 30 minutes.

Absorbance (595 nm)
.2
.1
.0
30
10
2
0
Time (min)

6.8. MEASUREMENT OF SODIUM AND POTASSIUM LEVELS

Sodium and Potassium levels in biological fluids were kindly measured by the Dept of Biochemistry and Chemical Pathology, Flinders Medical Centre. Measurements were made with an Ion-selective electrode using an automated Beckman Astra-8 analyser.

6.9. ADMINISTRATION OF RADIOISOTOPES IN PHYSIOLOGICAL STUDIES

In a number of studies, one or more radioisotopes were administered to animals to monitor changes in concentration of extracellular fluids. The five radio-labelled tracers used were ^{3}H-Water (TOH), ^{3}H-Testosterone, ^{14}C-Mannitol, ^{125}I-Normal Rat Plasma, and ^{22}Na-Sodium Chloride. Equilibration times allowed for markers to partition between vascular and extracellular compartments were based on descriptions by Chandrasekhar et al (1986) for Testosterone; and Cowie et al (1964), Setchell et al (1969), and Setchell and Sharpe (1981) for TOH, ^{22}NaCl, ^{125}I-NRS, and ^{14}C-Mannitol.

6.9.1. ^{3}H-Water

Tritiated water (100 uCi/ml) was administered by intraperitoneal injection of 1 ml. Equilibration for at least 3 hours was ensured, with some animals allowed overnight equilibration, prior to sampling of any fluids.

6.9.2. ^{3}H-Testosterone

Labelled testosterone was administered by the radiolabelled steroid infusion technique of Horton and Tait (1966). A Teflon cannula was placed into the jugular vein, under sodium pentobarbitone anaesthesia. A priming dose of 0.5 uCi of 1,2,6,7 ^{3}H-Testosterone (specific activity 370 uCi/ug) in 0.25 ml of 8% ethanol saline was injected into the jugular. Half an hour later, a constant infusion of ^{3}H-Testosterone was started (0.5 uCi/ml in 8% ethanol saline) with a Harvard infusion pump (Harvard Apparatus Co., Model 1100, Millis, USA) fitted with a 2 ml infusion syringe, at the rate of 18 ul/min into the same jugular vein. This infusion was maintained for two hours initially, to achieve equilibration (see Chandrasekhar et al, 1986), and continued during any extended sampling procedures after equilibration.

6.9.3. ^{14}C-Mannitol

Because mannitol is rapidly cleared from blood by the kidneys, animals injected with this marker had their ureters severed under sodium pentobarbitone anaesthesisa, prior to administration of the mannitol. This ensures recirculation via peritoneal fluid. Following this procedure, 1 uCi ^{14}C-Mannitol in 250 ul physiological saline was injected intravenously. Two hours equilibration was allowed prior to sampling of any fluids.

6.9.4. ^{125}I-Normal Rat Plasma

Normal rat plasma was obtained from donor animals, and iodinated using the method employed for radioimmunoassay protein hormone iodination as described in 2.13.3.1. In this manner, ^{125}I-NRP was prepared at a final concentration of approximately 140 uCi/ml. Incorporation was monitored as described by Thorrell and Larson (1978), with some 88% of labelled protein recovered after TCA precipitation.

Iodinated-NRP (5 uCi) was administered by intraperitoneal injection. Equilibration time for ^{125}I-Albumin is at least 6 hours (see Cowie et al, 1964; Setchell and Sharpe, 1981), although diffusion time from plasma for 100% of plasma albumin is 16.6 hours (Landis and Pappenheimer, 1963; see also 6.9.5. below). Thus animals were injected with ^{125}-NRP in the evening of the day before sampling was required, to allow overnight (14-16 hours) equilibration, prior to sampling of any fluids.

6.9.5. ^{22}Na-Sodium Chloride

Landis and Pappenheimer (1963) have published data summarising the disappearance from the circulatory system of various lipid-insoluble substances which distribute primarily in extracellular fluid. Such substances leave the vascular system at rates which vary inversely with molecular size. Disappearance from plasma is accompanied by simultaneous appearance of the test molecules in tissue spaces, again at rates which vary inversely with molecular size. Sodium chloride equilibrates extremely rapidly with a half time

clearance of less than 2 minutes from rabbit arterial plasma (compare with albumin, as discussed in 6.9.4.). Godhino and Setchell (1975) reported a half-time clearance for $^{24}NaCl$ extraction from ram testicular capillaries of 1 minute. In addition, Bustamante (1985, see also Bustamante and Setchell, 1981) found a 60% extraction of $^{22}NaCl$ on a single pass of plasma through the rat testis. These two reports suggest a rapid equilibration of $^{22}NaCl$ between blood and interstitial fluid in the testis. In the present studies, labelled sodium chloride (2 uCi) was injected intravenously and equilibration was allowed for one hour before any fluids were sampled.

6.10. STATISTICAL ANALYSIS

All data is presented as mean $\pm$ standard error of the mean (SEM), unless otherwise stated. The probability of significant differences between groups was calculated by Student's t-test and treatment effects were analysed by Analysis of Variance (ANOVA) or Analysis of Covariance (ANCOVA).

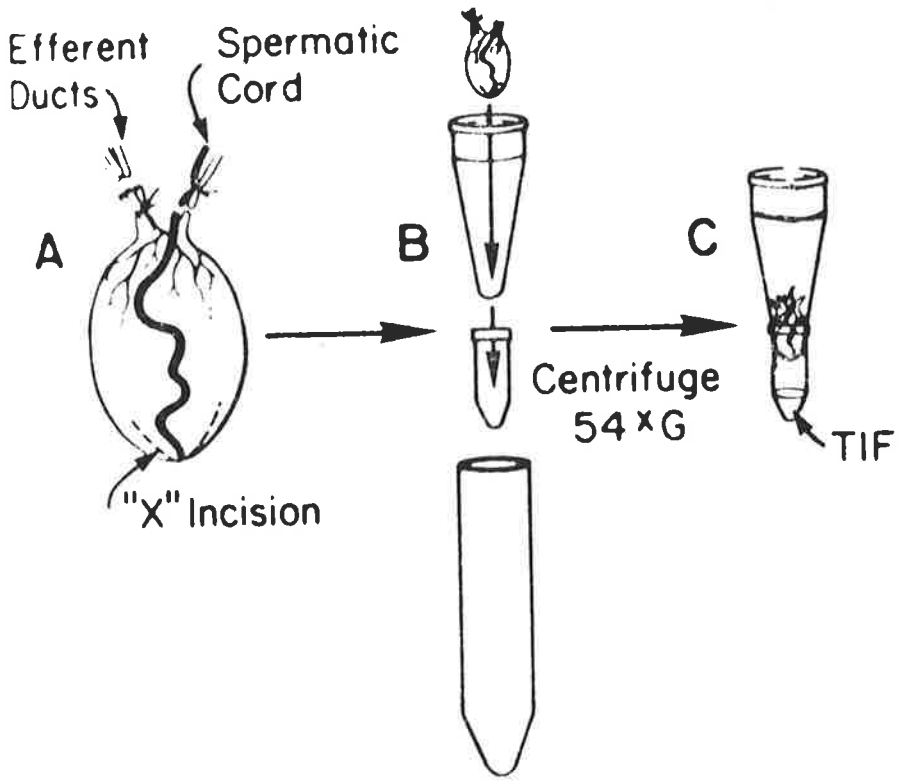
CHAPTER 7: INTERSTITIAL FLUID - A WINDOW ON THE INTERSTITIAL REGION

7.1. INTRODUCTION

With the discrepancy in the immune status of the interstitial region of the sheep and rat testis determined in the earlier experiments, it was of interest to examine interstitial fluids for factors that might be of consequence for allograft survival. In this regard, steroid hormone levels were of particular interest, since a number of steroids have been found to be immunosuppressive in vitro (see Kitzmiller and Rocklin, 1980; Stites and Siiteri, 1983) . To examine steroid levels in interstitial fluid requires the sampling of such fluid. The most widely used technique is that of Drip Collection (Pande et al, 1966), which was also detailed in the introductory review, and is seen in Figure 7.1. While the Drip system works well (in terms of fluid recovery) on a rodent testis, its application in other species is limited. This is probably due to the presence of the large lymphatic sinusoids in the rodent testis and the lack of the connective tissue that is found in the larger mammals such as sheep and pigs. However, as discussed in chapter 1, this technique has a number of other possible physiological short-comings, not the least of which is the necessity to remove the testis from its blood supply. This "fault" is shared by almost all techniques previously used. It is of concern that the testosterone levels reported for interstitial fluid collected by this technique are much higher than those found in testicular venous blood or testicular lymph (see Lindner, 1963, 1969; Wallace and Lascelles, 1964; Hagenas et al, 1978; Sharpe, 1979; Sharpe and Cooper, 1983; Sharpe et al, 1983; Turner et al, 1984). The close association of Leydig cells with blood capillaries (see McIntosh, 1969; and Chapter 1) must be of some physiological importance and yet

Figure 7.1. Drip-collection method employed by numerous investigators to obtain interstitial fluid from the testes of rats. Some investigators perform steps A and B, and then allow gravitational collection at 4°C for up to 17 hours or more. Others proceed on to step C, and use centrifugation at 54 x G at 0°C for 15 minutes to obtain the fluid exudate. Not to scale (adapted from Turner et al, 1984).

"Drip" Collection



the Drip collection system appears to assume that fluid formation and hormone secretion will continue to be physiologically normal for up to 17 hours at 4 ^{o}C without a blood supply.

For these reasons, it was decided to explore an alternative method of interstitial fluid collection.

7.2. THE PUSH-PULL CANNULA

7.2.1. Introduction

For some years, neurophysiologists were faced with the necessity to collect extracellular fluid continuously from the brain - a problem not dissimilar to that for testicular physiologists attempting to collect interstitial fluid. In 1961, J.H. Gaddum proposed the use of a "Push-Pull Cannula", which consisited of two concentric tubes. It was based on a system developed by Fox and Hilton (1958) for the perfusion of subcutaneous tissues between two parallel needles 1.5 - 2 cm apart. According to Gaddum (1961), the push-pull cannula had four distinct advantages (for neurophysiology) :

"(1) It detects the substances liberated under fairly normal conditions.

(2) It gives an indication of the turnover, rather than the stores, and can be used to study the effects of factors such as nervous stimulation.

(3) It should be possible to localise the site of liberation of substances in such tissues as the central nervous system more precisely than by other means.

(4) The blood-brain barrier presumably does not intervene."

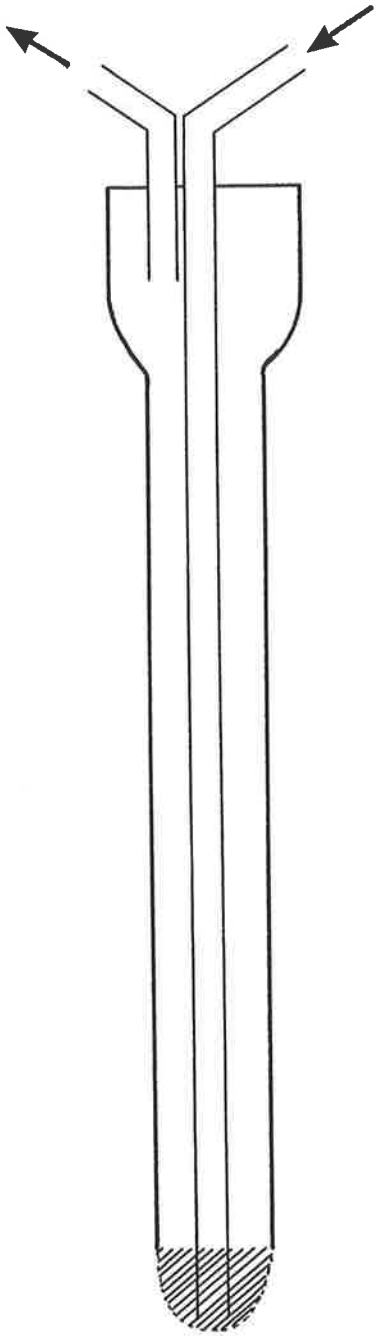
Push-pull cannulae have now been developed in various forms, and have been widely used in numerous applications. Phillipu (1984) has reviewed these applications, and summarises that "... probably the main advantage of working with push-pull cannulae lies in the versatility of the technique, ... The use of the push-pull cannula for detection of endogenous neurotransmitters in the perfusate (has) opened a new perspective in studying the dynamics of the release of neuroransmitters in distinct brain areas."

The similarity of the original problem in both disciplines, and the solution provided by the push-pull cannula for one of these disciplines, made this technique worthy of closer examination. The ability to measure endogenous substances in the perfusate obtained by a push-pull cannula was of obvious significance.

7.2.2. Construction

As already mentioned, the push-pull cannula originally described by Gaddum (1961) consists of two concentric tubes open at one end only (Figure 7.2.). The fluid is driven through the inner tube. The appropriate adjustment of the distance between the tips of the outer and inner tubes generates a siphon, which is important for the further transport of the fluid and for a constant rate of outflow as well. This technique has been extensively described (Szerb, 1967; Dudar and Szerb, 1970; Myers, 1972; Yaksh and Yamamura, 1974). Szerb (1967), Dudar and Szerb (1970), Yaksh and Yamamura (1974) and Honchar et al (1979) have thoroughly examined the experimental conditions necessary for a constant rate of flow, and for the recovery of substances

Figure 7.2. Section of Gaddum's push-pull cannula. Arrows indicate direction of flow, and the hatched area is the area between the perfusate and tissue. The outer tube is a No. 20 or No. 18 needle, the inner tube a No. 28 needle (from Phillipu, 1984).



diffusing from the tissue into the push-pull cannula. Of most importance is the relationship of the two components of the cannula. Since the lower tip of the inner cannula protrudes, an artificial cavity is created around the tip (Figure 7.2.). The outflow contains substances that were present in this cavity. To reach a constant flow rate, two pumps are needed : one to push the fluid into the cannula and the other to pull the perfusate from the outer tube into the collecting tube. Recovery from the perfusate of substances present in the tissue is good, provided that the tip of the inner tube protrudes to some extent (Phillipu, 1984). Indeed, the reproducibility of the results depends to a great degree on the position of the inner tube (Szerb, 1967; Dudar and Szerb, 1970; Yaksh and Yamamura, 1974). However, the greater the distance between the tips of the inner and outer cannulae and the higher the rate of perfusion, the more difficult it is to obtain a constant rate of outflow (Yaksh and Yamamura, 1974).

Two major problems were perceived with the designs used by neurophysiologists. Firstly, the dead space in the outer cannula (see Figure 7.2.) might affect reproducibility between samples. Secondly, the commercially available cannulae (kindly provided by Dr Richard Dyer, ARC Institute of Physiology, Babraham, Cambridge, U.K. and available from Clark Electromedical Instruments, Pangbourne, Reading, U.K.) were designed to allow the inner cannula to be unscrewed and removed from the outer cannula. While this is important for the introduction of such cannulae into brain, it also creates the potential for different distances to arise between the tips of the inner and outer cannula between different samplings, unless the components are screwed together to the same position each time. In the

studies to be presented in this thesis, push-pull cannulae were constructed by adapting stainless steel needles. 19G needles were used for the outer cannula and 25 G needles for the inner cannula. In both cases, luer-lock fittings were removed, and the sharp, pointed ends were removed and squared (see Figure 7.3.). The first problem described was overcome by introducing the inner cannula through the side of the outer cannula. In this way, suction on the outer cannula would draw perfusate up from the "cavity" directly out to the sampling point. The second problem related to separation of the two component cannulae. This separation was not required for the present studies, and thus the permanent fixing of the two cannulae (soldering of the inner cannula to the outer cannula at the point of entry) overcame this difficulty and ensured constant results for each cannula constructed. The inner cannula was allowed to protrude past the outer cannula for 1.0 mm. This allowed a perfusion surface area of 2.8 mm^3 around the cannula tip, calculated by the method of Szerb (1967) as seen in Figure 7.4.

7.2.3. Apparatus

The apparatus used to sample fluid from testes by the push-pull cannula is shown in Figure 7.5. For each cannula, the infusate was pumped into the inner cannula (see Figure 7.3.) with a Minipuls II peristaltic pump (Gilson, France) with a slow head. Connection between the pump and the cannula was made with polyvinyl tubing (I.D. 0.8 mm, O.D. 1.2 mm). The outer cannula was connected by Teflon catheter (I.D. 1.0 mm, O.D. 1.6 mm) to a 2 ml glass syringe on a Harvard Infusion pump (Model 1100, Harvard, Minnis., USA) for perfusate withdrawl. Both

Figure 7.3. A diagrammatic representation of the push-pull cannula in a testis, demonstrating the principles of mixing and diffusion across the perfusate interface and resultant dilution of collected fluid.

The Push-pull Cannula

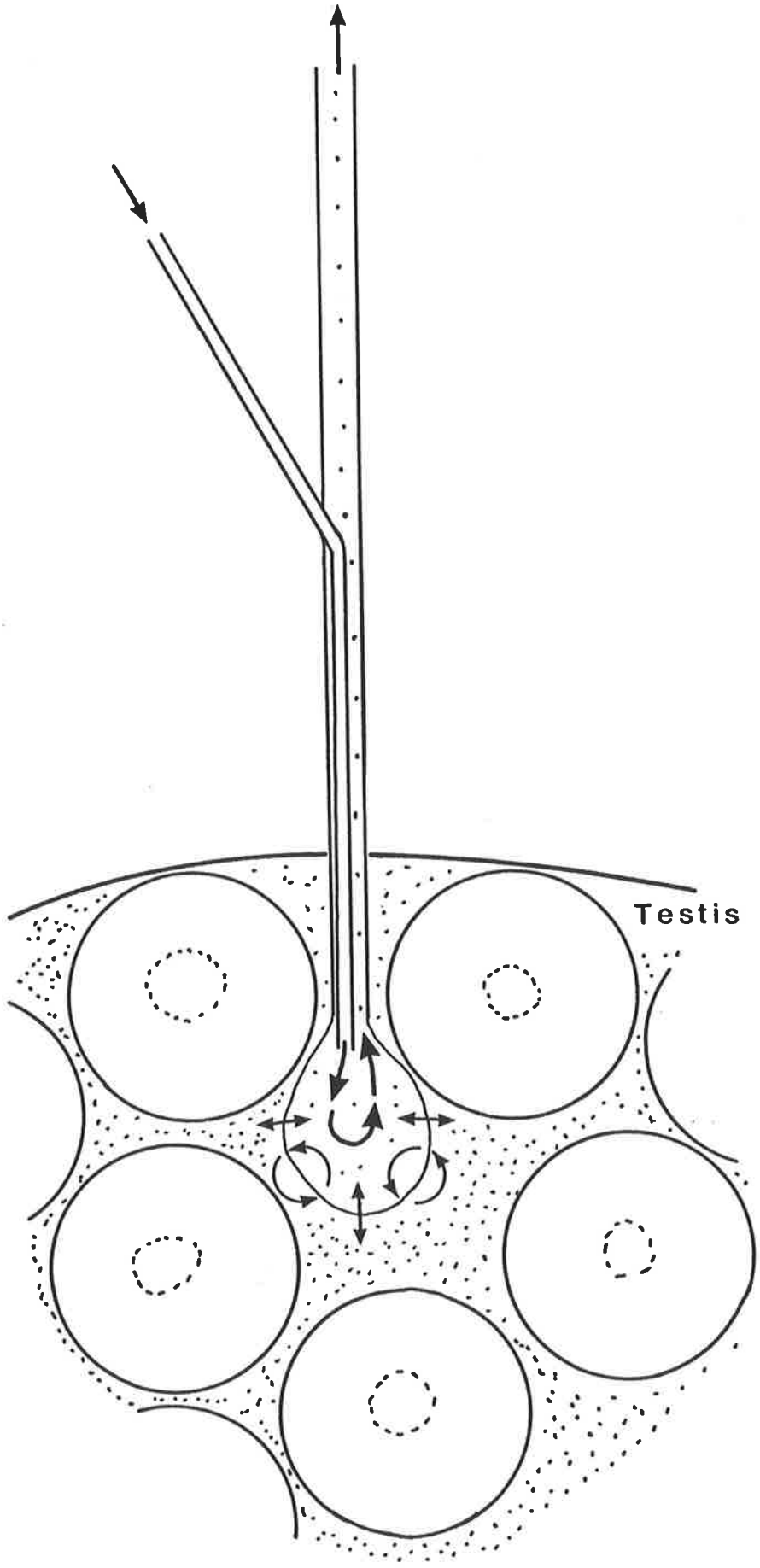
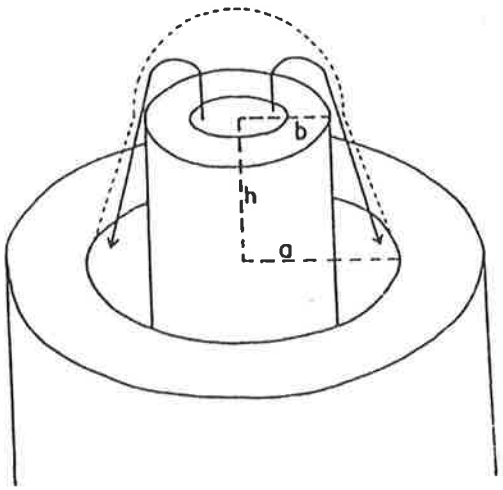


Figure 7.4. Method of calculation of perfusion surface area of a push-pull cannula as described by Szerb (1967). The diagram shows the tip of the cannula, and the measurements made for calculation; arrows indicate direction of flow, and the broken line shows the cross section of assumed interfacial area.



$$\text{Surface Area} = [\, 2\pi\,(b + 0.025)^2] + [\, a\pi\sqrt{a^2 + (h + h')^2} - (b + 0.025)\pi\sqrt{(b + 0.025)^2 + (h')^2}]$$

where :

a = inside radius of outer needle = 0.45 mm

b = outside radius of inner needle = 0.25 mm

h = protrusion of inner needle = 1 mm

h' = [(b+0.025)h]/[a-(b+0.025)] = 1.571

Surface Area of constructed cannulae = 2.788 mm^2.

Figure 7.5. Arrangement of apparatus used to sample interstitial fluid from rat testes with a push-pull cannula.

W = Water bath used to maintain stage (S) temperature.

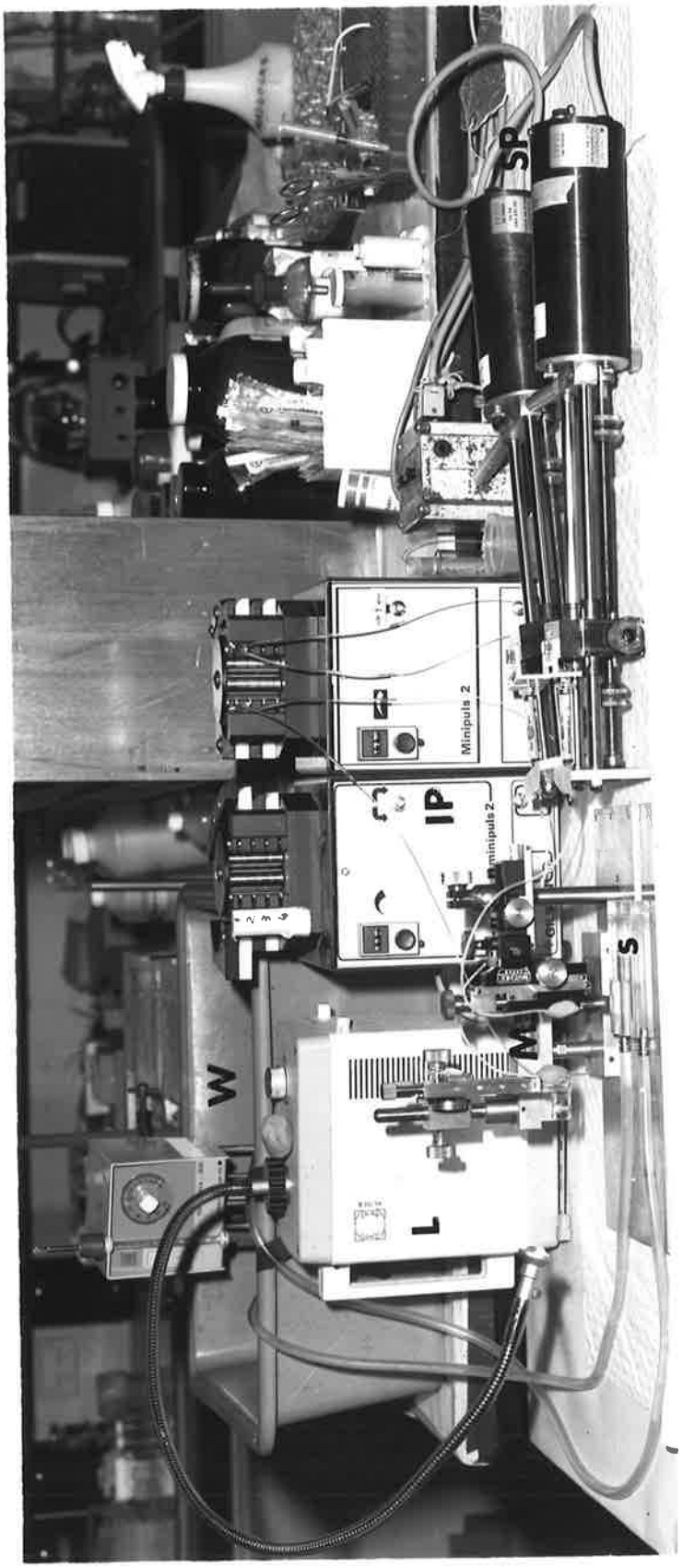
L = Cold-light source.

M = Micro-manipulators used to position push-pull cannulae.

S = Water-jacketed perspex stage used to hold testes during cannulation and sampling.

IP = Infusion pumps used to infuse perfusate through cannulae. One was a high-revolution pump for loading and flushing of the cannulae, and perfusion and sampling lines; and one was a slow-revolution pump for constant low-level infusion during sampling.

SP = Syringe pumps used to withdraw fluid from the cannulae.


SP
IP
Minipuls 2
Minipuls 2
W
M
L
S

pumps were adjusted such that infusion and withdrawal equilibrated at approximately 10 ul/min. The cannulae were attatched to micromanipulators, using plasticine (see Figure 7.6.a). A perspex stage was constructed to hold the testes for cannulation (Figure 7.6.a), and warm water was recirculated from a heated water bath through the stage, with water temperature adjusted to maintain testis temperature on the stage at normal scrotal temperature (approximately 33 ^{o}C; Kormano, 1967c).

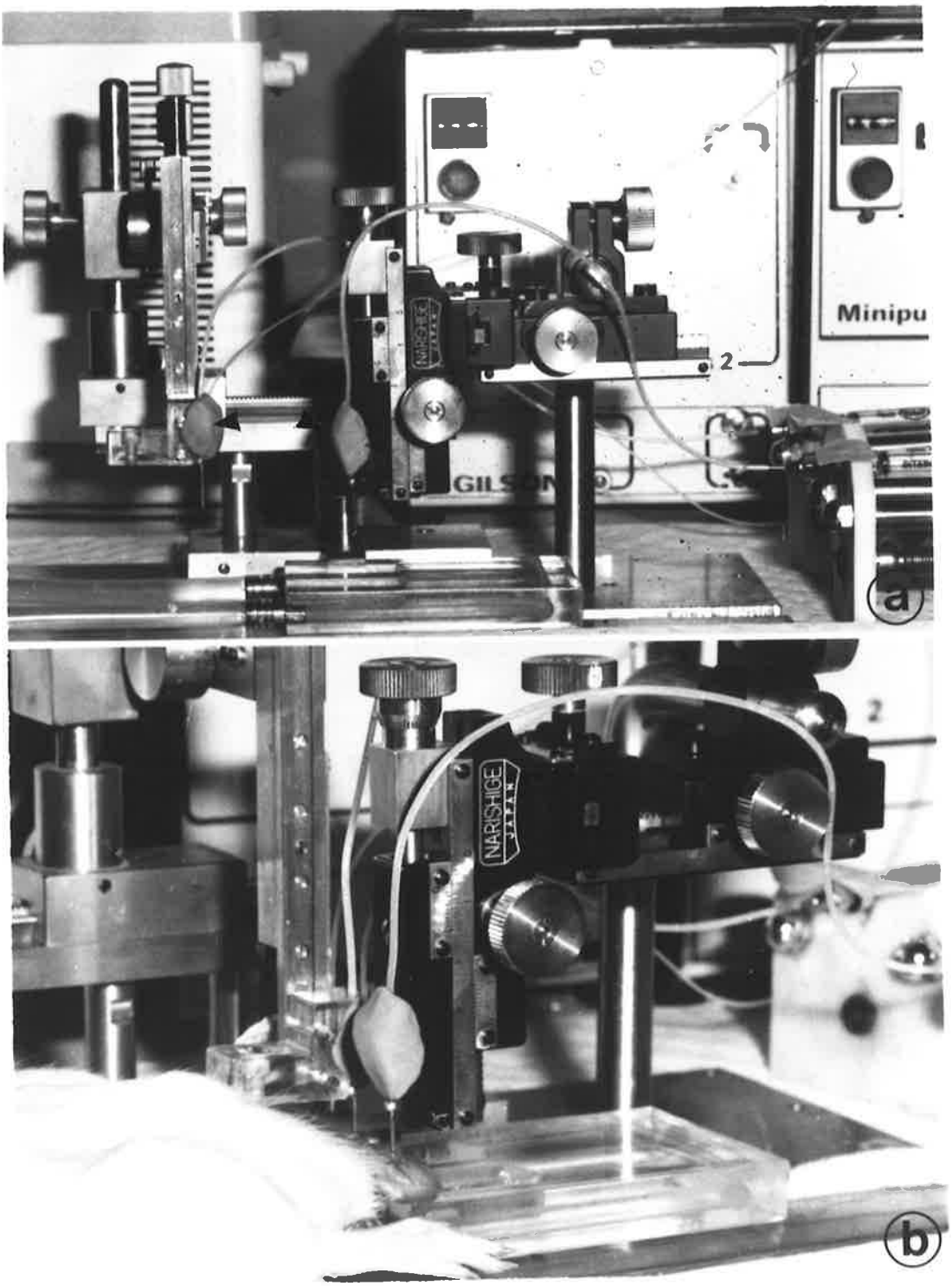
7.2.4. Use of the Push-pull cannula

Rats were anaesthetised with sodium pentobarbitone and each testis exposed through an incision in the scrotum. Animals were then positioned with their testes placed into the two grooves in the pre-warmed, perspex stage, with their tail under the stage (Figure 7.6.b). A small entry hole was made in the tunica of the testis to be cannulated, using a 25 G needle, and avoiding all obvious blood vessels. By carefully inserting a pair of pointed forceps, this hole was kept patent while the cannula was inserted using the micromanipulator. The forceps were withdrawn, so that the tunica enclosed the outer cannula. The cannula was inserted to a depth of approximately 5 mm, and Figure 7.6.b shows a cannula in position in a rat testis (see also Figure 7.3.).

7.3. INVESTIGATIONS IN THE RAT

As mentioned in the introduction to this chapter, the primary aim of these studies was to examine steroid levels in interstitial fluid,

Figure 7.6. (a) Close-up view of micro-manipulators with cannulae held in position with plasticine (▶). (b) Cannulae in position in the testes of a rat which have been withdrawn from the scrotum and placed into the grooves in the perspex stage.


Minipu
2
GILSON
a
NARISHIGE
JAPAN
2
b

and testosterone was chosen as the steroid to be measured in initial studies. To monitor physiological aspects of the technique, measurements of protein, sodium and potassium were also made.

7.3.1. Experimental Procedure

7.3.1.1. Application in the testis

As outlined in the discussions on the theory behind the push-pull cannula, the assumption in use is that components in the fluid in which the cannula is placed will cross the interface with the infusate around the extended tip of the inner cannula, and appear in the sampled perfusate. This has been found to occur both in vitro and in vivo in brain (see Phillipu, 1984). It was important to examine the performance of this theory in the testis. It was also important that perfusion would reach throughout the interstitium, and not simply flush out a particular region. This was checked by infusing a solution of Pontamine Sky Blue dye (0.5% in isotonic mannitol) through a push-pull cannula positioned in a testis.

To monitor the extent of tissue damage caused by cannulation of the testis, a testis was processed for histological examination after a push-pull cannula had been in place for one hour.

7.3.1.2. Measurement of Dilution

It was apparent from the literature, and the initial studies using the push-pull cannula in the testis that a certain amount of dilution occurs with this process. It would obviously be important to

monitor this effectively if actual levels in the original fluid are to be assessed.

A common technique for measuring fluid dilution in physiological studies, is to administer a radioactive tracer to an animal, and allow it to equilibrate between blood and the fluid compartment of interest. Assuming that tissue barriers do not affect equilibration, the levels of radioactivity should be the same in blood and collected fluid. The difference in levels of radioactivity measured in samples of blood and diluted fluid will provide a measure of the dilution occurring during collection. This technique was used to measure the amount of dilution of interstitial fluid collected with the push-pull cannula. Initially, tritiated water (^{3}H-Water) was used to monitor dilution in push-pull samples since this tracer is widely used in metabolic turnover studies. Administration and equilibration time were as discussed in 6.9.1. Following these studies, it was decided to also use ^{14}C-Mannitol, ^{3}H-Testosterone, and ^{125}I-labelled normal rat plasma to monitor dilution. In latter experiments, ^{22}Na-Sodium Chloride was also used. These tracers were chosen because of their similarity to compounds being used in the push-pull technique (see 7.3.1.3.) or their similarity to compounds of interest in interstitial fluid (see 7.3.). Administration and equilibration times were as discussed in section 6.9. Levels of radioactivity where monitored in samples by gamma (LKB 1271 Riagamma) or scintillation (LKB 1215 Rackbeta II) counting as appropriate to the tracer used. While some animals were injected with only one tracer, other animals received "cocktail" injections of two tracers. In these situations, samples were counted for each tracer individually, and corrected for cross-channel contamination as determined from standards of each tracer counted separately on all

channels.

7.3.1.3. Choice of Infusates

To monitor steroids, proteins, and sodium and potassium levels in the collected perfusates, it was necessary to use a medium that would not influence these results. At the same time it was important to use an infusate that would not be physiologically distressing to the testis. Two fluids were chosen for study. Isotonic Mannitol was chosen because it lacks all of the compounds of interest. Castrate Rat Plasma was also chosen for infusion, as a substance that might be more like that filtered by testicular blood vessels to produce interstitial fluid.

7.3.1.4. Protocol used

Following administration and equilibration of tracer(s), animals were re-anaesthetised as needed, and a push-pull cannula placed into each testis as required. Push-pull sampling was routinely performed for one hour, timed for each cannula from the time of insertion into the testis. After sampling, interstitial fluid was collected from the holding syringe and collecting cannula by backflushing down the line after removal of the push-pull cannula from the testis. Testicular venous and peripheral vena cava blood samples were obtained as described in section 2.3.5.1. All samples were immediately centrifuged. Aliquots of supernatant were sampled for measurements of radioactivity, and the remaining volumes stored at -20 ^{o}C until assayed.

Levels of Testosterone were measured in all samples collected, as described in 6.6.1. In those samples obtained with mannitol infusate, protein levels were measured in interstitial fluid and testicular venous plasma as described in 6.7. These samples were also monitored for sodium and potassium levels as detailed in 6.8.

7.3.2. Results

7.3.2.1. Application in the testis

Infusion of dye through the push-pull cannula resulted in a generalised colouring of the testis as seen in Figure 7.7. While there was a greater concentration of dye in the region closest to the point of infusion, there was evidence of more widespread distribution. The collected perfusate was more dilute than that initially infused into the cannula (Figure 7.7.). These results suggested that transfer of fluid was occuring from the cannula into the testis, and vice versa.

The histological examination did not reveal any significant damage to the testis. As seen in Figure 7.8. the cannula appears to separate the tubules along natural sheer-planes, with most structures left intact. Any damage is limited to the immediate vicinity of the cannula and neighbouring tubules appear quite normal.

7.3.2.2. Measurement of Dilution

Table 7.1. shows the amounts of various radioactive markers appearing in the push-pull perfusate, collected with either isotonic mannitol or castrate rat plasma infusate. The results indicate that

Figure 7.7. Demonstration of the mixing and diffusion occurring between perfusate and interstitial fluid in the testis with the use of the push-pull cannula. The vial in the upper left of the picture contains the Pontamine Sky Blue/Mannitol infusate, and the vial in the upper right contains the sample collected from the push-pull cannula. The lower left of the photogragh shows a control testis. The lower right of the photograph shows a testis after one hours sampling of fluid with the push-pull cannula using the PSB/Mannitol infusate. Note the distinct blue colouring over a large area of the cannulated testis, and the dilution of the dye colour in the sampled fluid compared that infused. This suggests that the perfusate is mixing with interstitial fluid throughout the testis, and that diffusion across the interstitial fluid/perfusate interface does occur.

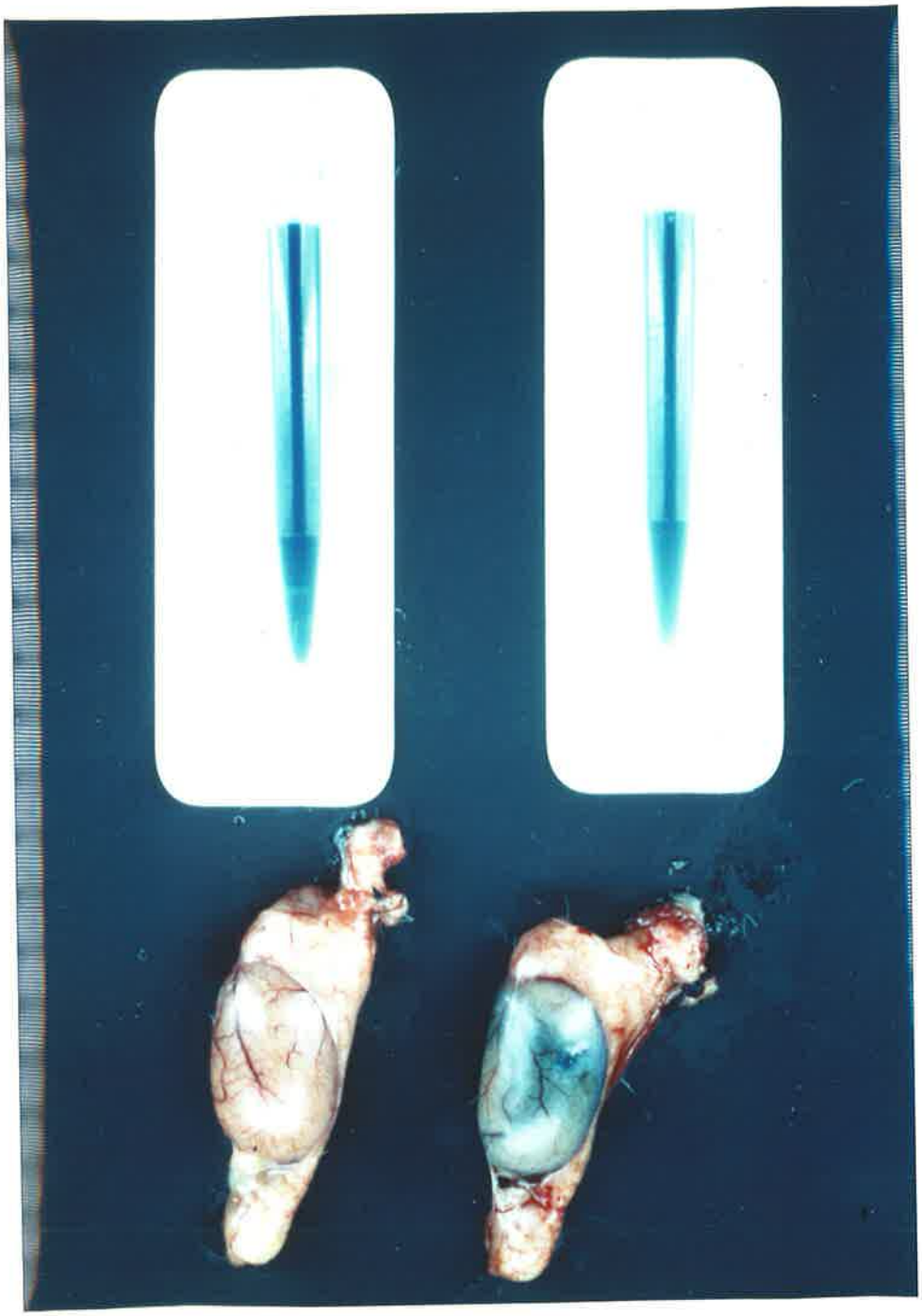


Figure 7.8. Sections from the testis of an adult rat after insertion of a push-pull cannula. (a) shows the point of entry of the cannula, and the separation of seminiferous tubules after cannulation (X 30). Some tissue debris is seen in the cannula hole. However, tissue damage is very localised and as seen in (b), tubules seem to have parted along natural sheer-planes (X 80). The cannula was coated in indian ink prior to insertion, and particles of ink can be seen in this region. The relationship of tubules and interstitial cells in regions surrounding the point of cannulation appears quite normal. As seen in (c), tubules separated from their neighbouring cells by the push-pull cannula appear intact, and have maintained their structure (X 320). (d) shows particles of indian ink which have been carried away from the site of cannnulation (X 320). These particles are seen between two tubules in close aposition, which is evidence for the ability of substances to move readily through the interstitium of the rat testis.

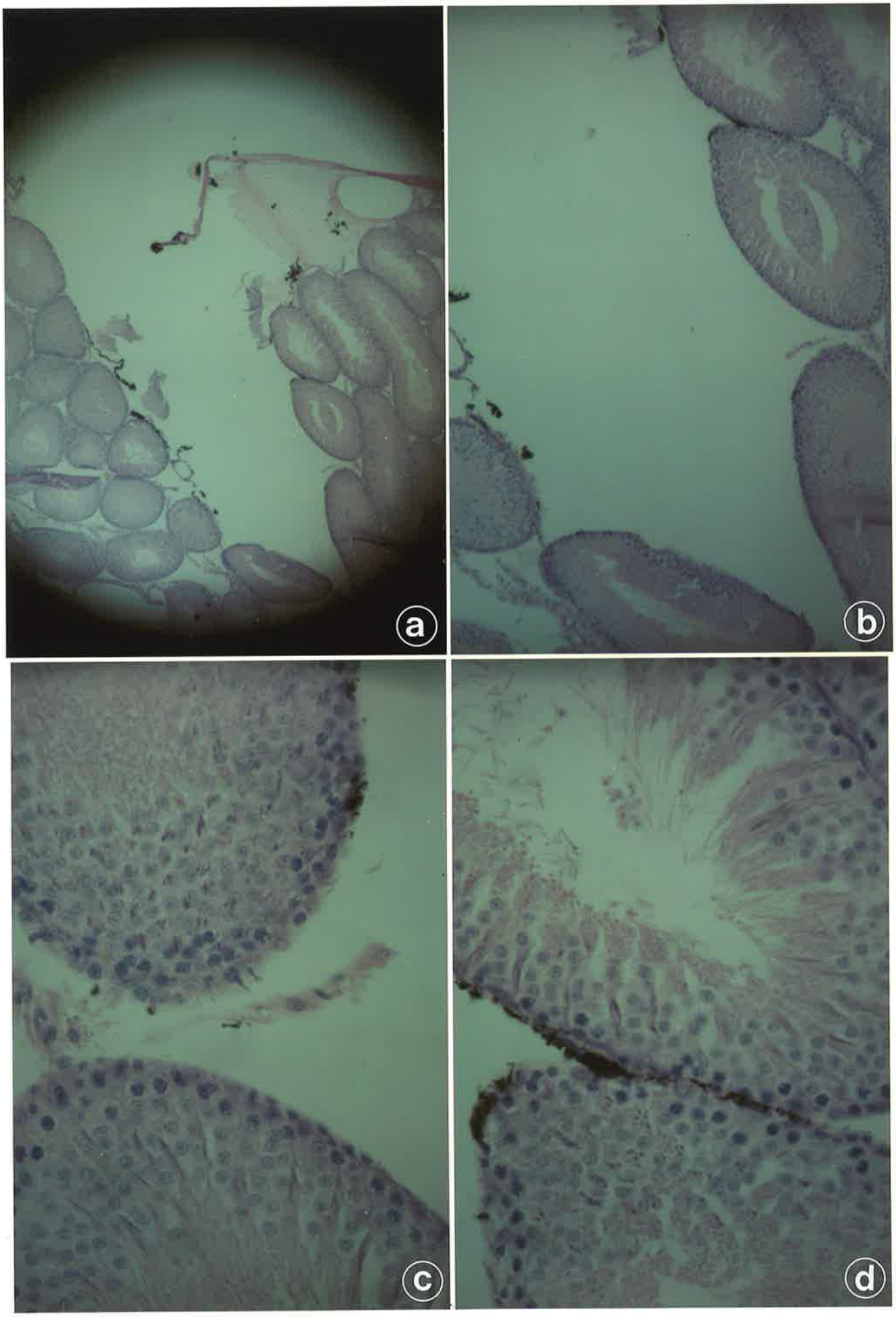

a
b
c
d

Table 7.1. Amounts of various radioactive markers appearing in the effluent from a push-pull cannula introduced into the testis of an anaesthetised rat. Values are expressed as a percentage of levels in testicular venous blood sampled at the same time, and are presented as means with the standard errors of the means, and the number of observations.

	Infusate used	
	Isotonic mannitol	Castrate rat serum
^{3}H-water	17.1 ± 3.2% (8)	3.92 ± 0.61% (5)
^{14}C-mannitol	1.76 ± 0.38% (4)	1.02 ± 0.32% (3)
^{3}H-testosterone	6.19 ± 2.07% (4)	2.75 ± 1.51% (3)
^{125}I-rat serum protein	0.82 ± 0.14% (4)	0.20 ± 0.11% (4)
^{22}NaCl	5.16 ± 1.21% (10)	-

the amount of radioactivity entering the fluid passing through the cannula depends on both the marker, and the type of infusate. While TOH passed rapidly into mannitol infusate, it did not transfer so readily into castrate rat serum. This relationship held true for all other markers ($P < 0.05$).

7.3.2.3. Analysis of Interstitial Fluid

Having determined levels of testosterone, protein, sodium and potassium in perfusates and blood samples, corrections had to be made in the push-pull collections for the dilution created with the technique. The results discussed in 7.3.2.2. indicated that it was necessary to correct for the dilution in a collection using a marker appropriate to the substance of interest. When the concentration of albumin in interstitial fluid was calculated using the dilution of iodinated rat plasma protein into isotonic mannitol, values similar to blood plasma were obtained (Table 7.2.). When the concentration of testosterone in interstitial fluid was calculated using the dilution of ^{3}H-testosterone into either mannitol or castrate rat plasma, the values obtained were lower than testicular venous blood values measured under the respective treatments. While the levels calculated from castrate rat plasma infusates were significantly higher than those obtained with mannitol infusates, the venous blood levels were also significantly higher in these animals (Table 7.2.). To ensure that these results were not biased by an unequal distribution of ^{3}H-testosterone marker, ^{3}H-testosterone levels were measured in blood and drip-collected interstitial fluid following infusion of the marker as described in section 6.9. No significant difference ($P < 0.01$, $n=4$) was found in the levels of marker in any of the samples collected. Sodium levels in interstitial fluid were corrected for dilution of ^{22}NaCl into mannitol infusate. While no direct marker was available for measurement of potassium dilution, it was assumed for calculations that sodium and potassium ions would diffuse at similar rates, and

Table 7.2. The calculated concentrations of protein, testosterone, sodium and potassium in testicular interstitial fluid, with measured values for testicular venous plasma. Values are presented as means with the standard errors of the means, and the number of observations.

	Interstitial fluid, collected using		Testicular venous blood plasma	
	Isotonic mannitol	Castrate rat serum	Isotonic mannitol	Castrate rat serum
Protein (mg/ml)	75.3 ± 12.9 (4)	-	58.4 ± 2.8 (5)	-
Testosterone (ng/ml)	11.6 ± 4.2 (9)	233 ± 60 (8)	23.4 ± 5.2 (7)	591 ± 68 (9)
Sodium (mmol)	141.7 ± 18.2 (9)	-	137.8 ± 1.9 (9)	-
Potassium (mmol)	12.2 ± 2.5 (9)	-	4.4 ± 0.1 (9)	-

thus potassium levels in interstitial fluid have been corrected for dilution against $^{22}NaCl$. The results suggest that interstitial fluid contains similar amounts of sodium but proportionally more potassium than blood plasma (Table 7.2.).

7.3.3. Discussion

These results suggest that the levels of testosterone in interstitial fluid are no higher than those of testicular venous blood - a finding that is in direct contrast to those reported by workers using the drip collection method (Table 7.3.). The values calculated from the mannitol infusates may be falsely low because of the low binding capacity of this fluid. The dilution factor is all important in these studies in determining the final concentrations of substances in push-pull perfusates, and calculation of absolute concentrations with push-pull collection may need refinement. The validation of the dilution correction and the reasons for using ^{3}H-testosterone to determine dilution correction for testosterone levels have already been discussed. However, the disparity in final levels determined, in comparison to reports by other authors using other techniques (see Table 7.3.), requires careful consideration. It cannot be entirely attributed to error of dilution correction, although some error must no doubt be allowed. It is of interest that when castrate rat serum was infused instead of mannitol, testosterone levels in the collected fluid increased significantly. This may mean that protein is important for testosterone transport, and that protein should be added to the perfusion medium. Indeed, Ewing et al (1979) have suggested an important role for protein in testosterone transport. However, the ratio of testosterone in interstitial fluid / testicular venous blood was comparable to that obtained with mannitol infusate, which suggests that this is not a problem. That all values were significantly increased when castrate rat serum

Table 7.3. Testosterone levels in testicular interstitial fluid and testicular venous blood of male rats, with the different techniques used to obtain interstitial fluid. Values are presented as means with the standard errors of the means, and the number of observations.

Method	Testosterone levels (ng/ml) I.F.	Testis Vein	Reference
Micropuncture	150 ± 27 (17)	-	Comhaire and Vermeulen, 1976
Drip / Centrifuge	137 ± 25 (10) 73 ± 5 (26)	- 28 ± 5.3 (10)	Hagenas et al, 1978 Turner et al, 1984, 1985
Drip / Gravity	315 ± 29 (4) 590 ± 20 (8)	- 90 ± 20 (8)	Sharpe et al, 1983
Push-pull cannula	11.6 ± 4.2 (9)	23.4 ± 5.2 (7)	

was infused, was at first surprising. However, the high levels of gonadotrophins in castrate plasma may be important. LH levels were measured in the castrate plasma infusate of these experiments and were found to be significant (> 8 ng/ml). It would appear that the infusion of castrate rat plasma directly into the testis provided a significant gonadotrophin stimulus resulting in an increased secretion of testosterone. That the ratio between interstitial fluid and venous blood remained similar to that in the unstimulated testis cannulations was indeed interesting, and suggests that Leydig cells may in fact secrete proportionately more testosterone directly into blood vessels than into interstitial fluid. The results also suggest that the much higher testosterone concentrations in testicular interstitial fluid compared with testicular venous blood reported previously (Sharpe et al, 1983; Turner et al, 1984, 1985) may be artefacts due to the unphysiological techniques used for collecting the interstitial fluid.

Previous measurements of total protein in drip collected fluid have been variable. Pande et al (1966) found levels in rat fluid to be almost twice those of serum, and levels in human fluid to be almost half those of serum (Pande et al, 1967). The similarity in protein levels measured in interstitial fluid and venous blood in the present study confirms a previous report by Sharpe (1979). This is possibly of significance in determining the physiological usefulness of the technique. Yaksh and Yamamura (1974) reported protein levels in push-pull perfusates in brain to be a useful indicator of tissue damage with increased levels of protein reflecting some degree of tissue damage. The present results, in conjunction with the histological studies, would suggest that tissue damage in the testis

is negligible with the push-pull technique.

The potassium concentrations reported in drip collected interstitial fluid (Sharpe, 1979; Pande, 1966) are probably falsely high. Potassium levels in testicular lymph are comparable to blood plasma levels, although tubular levels are significantly greater than blood plasma levels (Setchell and Waites, 1975). However, the levels found in interstitial fluid in the present studies are still more than twice those of blood plasma. This is most surprising, and is so far unexplained. While it might be indicative of cell or tubublar damage, as already discussed the histological examinations have not revealed any extensive damage that might contribute to such levels.

7.4. Conclusions

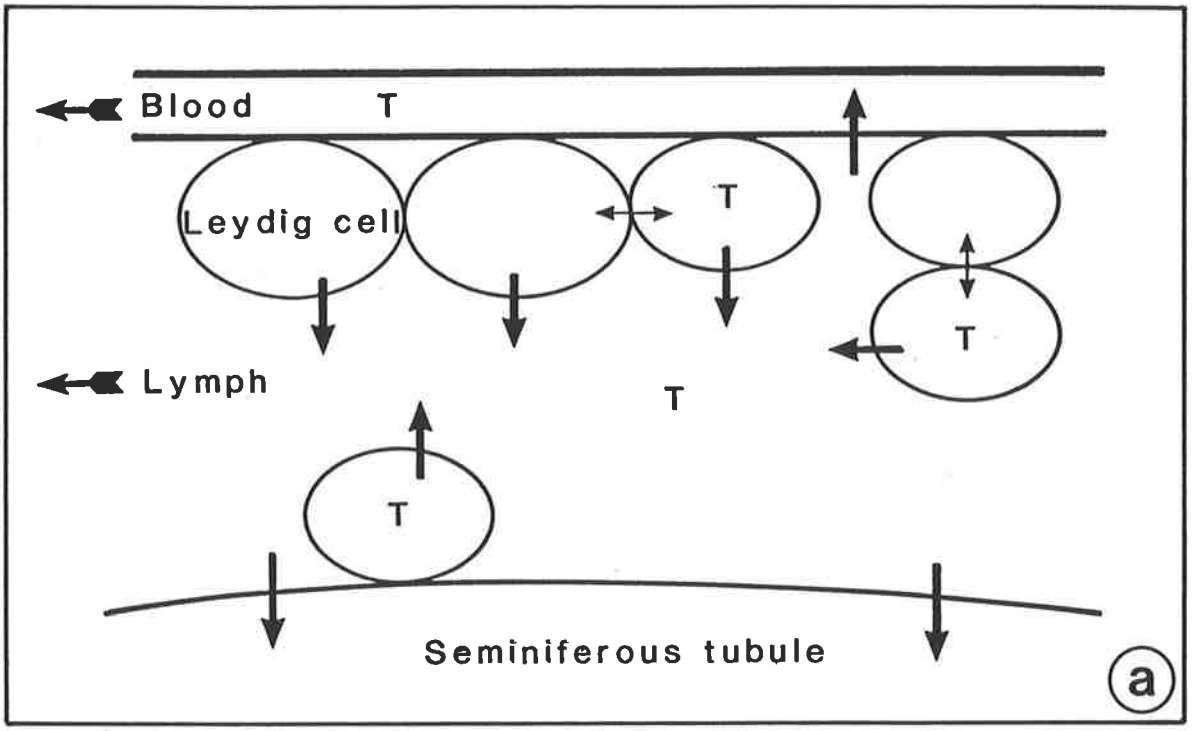
The results presented indicate that testosterone levels in interstitial fluid are unlikely normally to exceed testicular blood levels. Assuming that interstitial fluid is the fluid from which testicular lymph originates, this theory would account for the reports by Lindner (1963, 1967, 1969), Wallace and Lascelles (1964), and Setchell et al (1967), of testosterone levels in testicular lymph, being some 70 % of testicular venous blood levels. Buhl et al (1982) and Marshall et al (1984) have reported maintenance of spermatogenesis in hypophysectomised rats and stalk-sectioned monkeys given exogenous testosterone, when intratesticular levels of testosterone were 10 - 20 % of levels previously reported as normal. In 1979, Cunningham and Huckins reported the persistence of complete spermatogenesis in intact rats treated with testosterone propionate in which intratesticular levels of testosterone were 20% of normal. Rea et al (1986) have

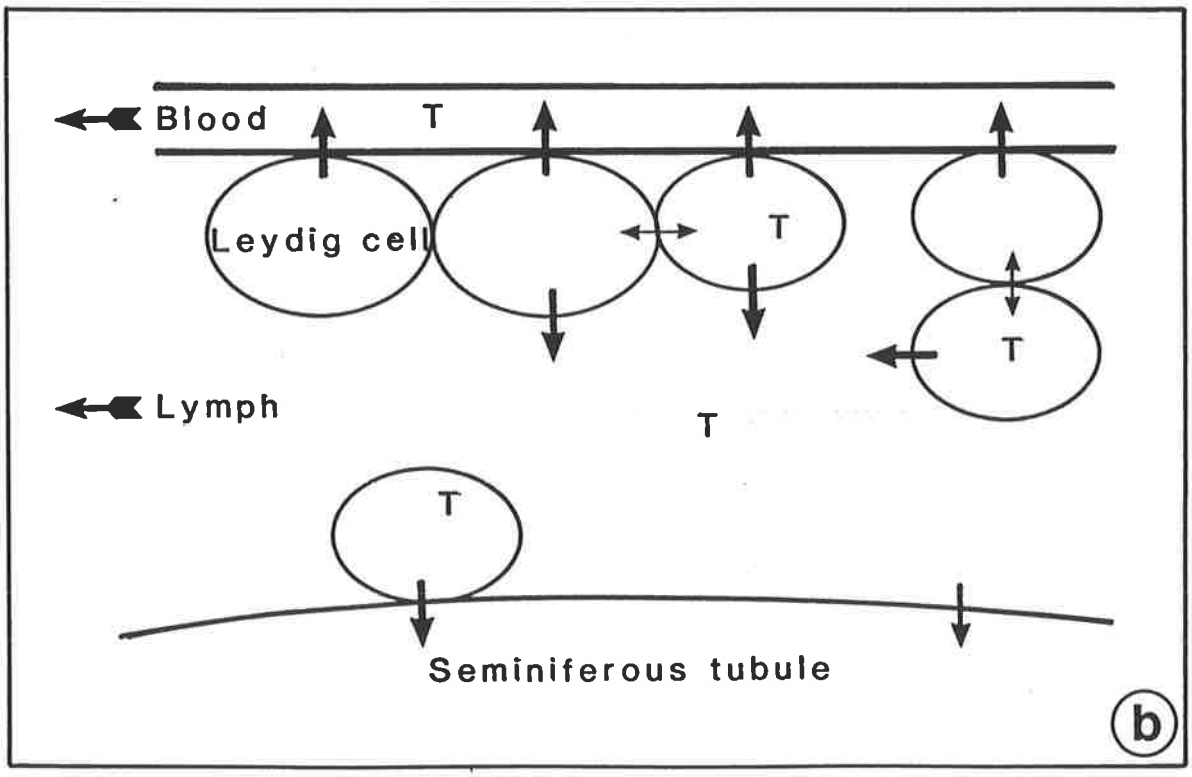
recently reported quantitative maintenance of spermatogenesis in hypogonadotrophic adult rats treated with testosterone implants, where intratesticular levels of testosterone were 15% of normal. The results from the present study suggest however, that the previously reported "normal" levels may have been overestimated by up to 10 times, and that the above studies (Cunningham, 1979; Rea et al, 1986) may in fact be measuring more normal levels of intratesticular testosterone. It is important to consider how this discrepancy might arise. As mentioned, the present study suggests that Leydig cells secrete testosterone more selectively into blood vessels than into interstitial fluid/testicular lymph, and this hypothesis is presented in Figure 7.9. In this model, interstitial fluid levels of testosterone are more a consequence of levels of testosterone in blood, and changes in blood flow and vascular permeability are thought to have dramatic effects on testosterone levels in interstitial fluid. This being the case, the drip collection system is faced with an immediate problem. Isolation of the testis from the blood supply would prevent this route of secretion. Physiologically, the Leydig cells in close proximity to blood vessels (and McIntosh, 1969 reported the majority of Leydig cells to be in such a relationship, see also Figure 1.2.) might indeed be affected by the lack of blood flow in these vessels, under these conditions. Assuming that they can continue to secrete testosterone normally (a questionable assumption indeed), the secreted product is unlikely to find its way into a blood vessel, and would more than likely accumulate in the "interstitial" fluid. The concentration of such secretion into interstitial fluid would elevate the concentrations measured in drip collected fluid to an abnormal level. It may be that values obtained from drip collected fluid

Figure 7.9. Diagrammatic representations of the proposed mechanisms of testosterone secretion in the rat testis.

(a) The existing hypothesis proposes that the Leydig cells primarily secrete androgen into the extravascular interstitial fluid (arrows). This creates a high concentration of androgen in this fluid which supplies the seminiferous tubules with the high levels needed for spermatogenesis. Blood levels of testosterone arise from the diffusion of testosterone from the intertitial fluid into the blood capillaries. Some mechanism must also exist to restrict the amount of testosterone secreted in testicular lymph, since the levels of testosterone measured in this fluid are no greater than venous blood levels (see text for discussion).

(b) The new hypothesis proposed from the work of this thesis. Leydig cells preferentially secrete testosterone into blood vessels (arrows). Some Leydig cells will secrete directly into interstitial fluid, and some may supply seminiferous tubules directly. However, interstitial fluid levels of testosterone are comparable to blood levels and arise more as a consequence of blood flow rate, and capillary permeability. These factors appear to be carefully regulated by the seminiferous tubules, and cells in the interstitial tissue.

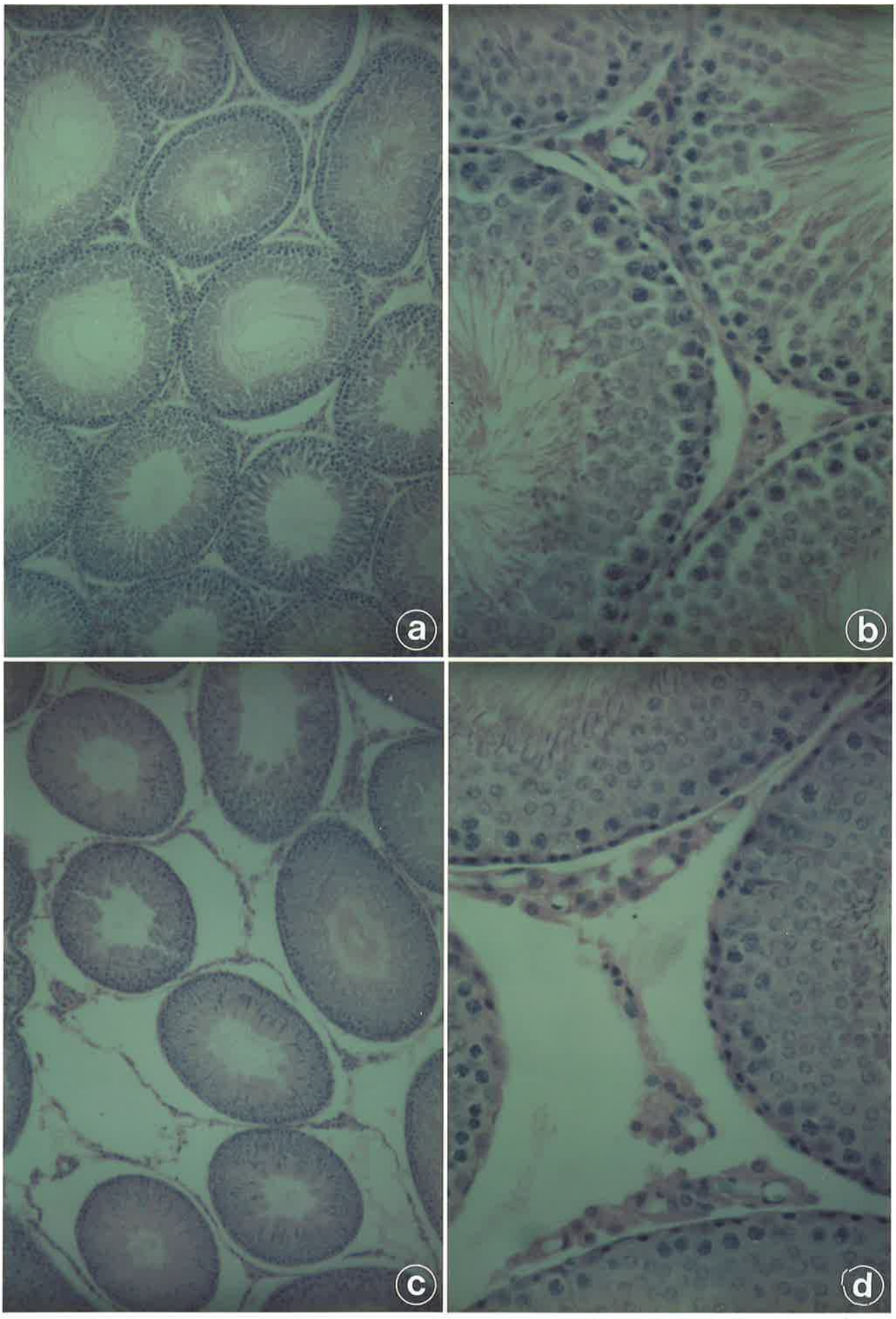

Blood
T
Leydig cell
T
T
Lymph
T
T
Seminiferous tubule
a


Blood
T
Leydig cell
T
T
Lymph
T
T
Seminiferous tubule
b

represent intratesticular "stores" of testosterone, rather than normal interstitial fluid levels. Figure 7.10. shows sections from a testis after drip collection was maintained for 18 hours. It is important to note that while the morphology is reasonably normal, a certain amount of shrinkage of the tubules has occurred during collection. This may affect intercellular communication and disturb normal physiological processes in the interstitial region.

All of these points raise serious doubts as to the usefulness of drip collection in physiological studies on interstitial fluid. It is also important to note that the push-pull system described here probably requires refinement. The process of dilution of the perfusate makes it unsuitable for some studies. The absolute levels of testosterone calculated depend on the dilution factor determined, and are probably subject to some error. However, the degree of difference in values obtained by push-pull collection compared to drip-collection cannot be accounted for simply by error of dilution correction. Further studies are required (see chapter 8), but this work has demonstrated that a re-evaluation of our understanding of the environment of the endocrine testis may be needed. Levels of steroids in interstitial fluid, and the mechanisms of secretion found in the endocrine testis also require further investigation.

Figure 7.10. Sections from the testes of an adult rat. (a,b) Control sections from a testis fixed by vascular perfusion at the time of castration (a - X 80, b - X 320). (c,d) Sections from a testis in which the blood was flushed from the vasculature with saline at the time of castration, and the tissue fixed by vascular perfusion after 18 hours of Drip-collection of interstitial fluid at 4 ^{o}C (c - X 80, d - X 320). Note the considerable shrinkage of tubules after drip-collection (c,d) when compared to controls (a,b). The cells in the interstitial region appear to lose a considerable amount of contact with the seminiferous tubules under such conditions, which may affect cell-cell communication and paracrine control.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CHAPTER 8: TESTOSTERONE LEVELS IN INTERSTITIAL FLUID - EFFECTS OF VARIOUS EXPERIMENTAL TREATMENTS

8.1. INTRODUCTION

This chapter reports on a series of experiments undertaken firstly to test the performance of the push-pull cannula, and secondly to examine the hypothesis of testosterone secretion proposed in the last chapter. Hypophysectomy with pregnenolone supplementation, treatment with hCG, treatment with EDS, heating of the testes, efferent duct ligation, and insertion of testosterone implants, were used to induce various changes in the testis, the interstitial fluid compartment, or in testosterone production/secretion. The push-pull cannula was used to obtain interstitial fluid from testes after these treatments, and compared with drip collection in some instances. Testosterone levels were measured in the collected fluid and in testicular and peripheral venous blood.

8.2. EXPERIMENTAL PROCEDURE

8.2.1. Effect of anaesthesia and push-pull cannulation on testicular venous concentrations of testosterone.

Levels of testosterone in testicular venous blood obtained from testes after push-pull collection of interstitial fluid were lower in the initial experiments than might normally be expected (see 7.3.2.3.). To investigate this further, six groups of four animals (330-500 g) were used to examine the effects of pentobarbitone anaesthesia and push-pull cannulation on testicular venous blood levels of testosterone. One group of animals was processed for each one of six treatments. This involved sampling testicular venous blood either immediately the animal was anaesthetised, one hour after

anaesthesia was induced with testes kept in the scrotum, one hour after anaesthesia was induced with testes exposed on the pre-warmed perspex stage detailed in 7.2.4., after one hour of sampling with the push-pull cannula, after two hours of exposure on the perspex stage, or after one hours exposure on the stage and one hours sampling with the push-pull cannula.

8.2.2. Hypophysectomy with pregnenolone supplementation

The importance of pituitary involvement in testosterone production has already been discussed in chapter 1. The gonadotrophin LH is necessary for a number of steps in androgen production, but ultimately acts on a specific step in the transformation of cholesterol to pregnenolone. It does not however, increase conversion of pregnenolone to testosterone (Hall, 1966). Supplementation of hypophysectomised animals with pregnenolone (1mg/100 g bw, daily for 14 days) maintains low peripheral plasma testosterone levels and normal rete testis testosterone levels (Harris and Bartke, 1975; Anthony et al, 1984).

Four groups of four animals (300-435 g) were hypophysectomised as described in 2.3.2. Pregenenolone in 0.1 ml of Sesame Oil, was administered to three groups at 0.5, 1.0, and 2.0 mg/100 g bw/ day for 14 days as described in 6.3.(see also Steinberger et al, 1975; Anthony et al, 1984). The fourth group was injected with the vehicle only. Injections were begun on the day of hypophysectomy. Interstitial fluid was collected from both testes with the push-pull cannula 24 hours after the last injection, as described in 7.2.4., and testicular

venous and peripheral blood collected as described in 7.3.1.4.

8.2.3. Human Chorionic Gonadotrophin

A single injection of hCG into a male rat has been shown to produce a striking increase in the permeability of the testicular blood vessels to albumin, in the amount of extracellular fluid in the testis, and in testicular lymph flow (see 3.5.3., and Setchell and Sharpe, 1981). In addition, testosterone concentrations in testicular venous blood and peripheral blood are increased at 2, 16 and 72 hours after such an injection (Hodgson and de Kretser, 1982,1984; Hodgson et al, 1983; Risbridger et al, 1981; Sowerbutts et al, 1986). It is likely however, that these responses are under different mechanisms of control (see 3.5.3.1.3.)

Twelve groups of four animals (300-450 g) were injected with 50 i.u. of hCG subcutaneously. Interstitial fluid was collected from a group of animals (from both testes) at 0, 2, 4, 8, 12, 18, 24, 30, 36, 48, 72, and 96 hours after injection, using the push-pull cannula as described in 7.2.4., and venous blood collected as described in 7.3.1.4.

8.2.4. Ethane Dimethane Sulphonate (EDS)

As already described in 3.6., EDS primarily injures Leydig cells. Rommerts et al (1985) have reported that EDS specifically inhibits the LH-regulated functional properties of the mature Leydig cell, possibly via alkylation of proteins. Molenaar et al (1985) have reported the

complete degeneration of Leydig cells by 72 hours after EDS injection, with no significant testosterone production detectable at this time.

Two groups of four rats (280-360 g) were used, with one group given intraperitoneal injections of EDS (75 mg/kg bw) dissolved in DMSO/water (1:3) as described in 2.8., and the other group injected with DMSO/water only. Three days later, interstitial fluid and venous blood were collected as described in 7.2.4. and 7.3.1.4.

8.2.5. Heat treatment

Heating the testis primarily causes damage in the seminiferous tubules, although not all cells there are damaged to the same extent (Collins and Lacy, 1969). The interstitial tissue appears hypertrophied after the testis has been heated or made cryptorchid. Indirect tests of androgen production suggest a moderate fall with sometimes a transient increase 10 to 15 days after heating (see Setchell, 1978). Kerr et al (1979) found cytological changes in Leydig cells of the cryptorchid testis that were suggestive of increased steroid secretion. However, peripheral levels were normal or lowered in these animals, suggesting a biosynthetic block in testosterone production, or conversion of testosterone by damaged tubules to other metabolites. The concentration of testosterone in the sera of rats whose testes had been locally heated to 43 ^{o}C for 30 minutes did not show any significant changes in a 7 week period following heating (Main et al, 1976; Setchell, 1978).

Eight groups of four animals (270-425 g) were subjected to

localised heating of the testes at 43 ^{o}C for 30 minutes, as described in 6.2. Interstitial fluid was collected from a group of animals (from both testes) at 0, 7, 14, 21, 28, 35, 49, and 63 days after heating, using the push-pull cannula as described in 7.2.4., and venous blood collected as described in 7.3.1.4.

8.2.6. Efferent Duct Ligation

Ligation of the efferent ducts of the rat testis causes disruption of spermatogenesis with maximum atrophy of the seminiferous epithelium occurring at 21 days after efferent duct ligation (Smith, 1962; Main et al, 1978; Main and Setchell, 1980; see also 3.5.1.). Collins et al (1978) and Collins and Tsang (1979) have reported the light microscopic appearance of the Leydig cells to be normal 21 days after efferent duct ligation, and their ability to produce androgens in vitro to be unimpaired. Main et al (1978) have reported FSH levels to increase from 5 to 21 days after duct ligation. Risbridger et al (1981b) found increased levels of serum LH two to four weeks after efferent duct ligation, along with an increased size of Leydig cells and an enhanced testosterone response to gonadotrophin stimulation in vitro despite a marked loss in LH-hCG receptors. Serum testosterone levels were not affected two weeks after either unilateral or bilateral ligation, remaining unaltered at four weeks in the unilaterally ligated animals but significantly decreased in the bilaterally ligated animals. The results suggest that changes in Leydig cell function after efferent duct ligation are due to local changes in the testis, probably involving the paracrine control system (Risbridger et al, 1981b).

In four groups of four animals (280-425 g) the efferent ducts of both testes were ligated (bilateral ligation) as described in 2.3.4. Interstitial fluid was collected from a group of animals at 0, 7, 14, and 21 days after ligation, using the push-pull cannula as described in 7.2.4., and venous blood collected as described in 7.3.1.4.

8.2.7. Testosterone Implants

Testosterone-filled silastic implants have been used in a number of studies to investigate the role of this hormone in the maintenance and restoration of spermatogenesis under a number of experimental conditions (Ahmad et al, 1973; Buhl et al, 1982). Depending on the length of these implants, serum testosterone levels can be suppressed slightly, or elevated 6 to 30 fold (Huang and Nieschlag, 1986). Sun et al (1986) have also investigated the effects of such implants on plasma testosterone, LH, and FSH levels, and on testosterone levels in interstitial fluid collected with the drip collection method. Huang and Nieschlag (1986) monitored changes over an 8 week period, and the majority of changes in testosterone and gonadotrophin levels seen after 8 weeks appear to have occurred within the 1st week of implantation.

Four testosterone-filled silastic implants per length of 2, 4, 8, 12, and 16 centimetres were prepared as described in 6.4. One group of four X 2 cm implants were not filled, and were used as controls. Six groups of four animals (400-580 g) were implanted with the capsules as described in 6.4. One week later, interstitial fluid was collected

from one testis in each animal, using the push-pull cannula as described in 7.2.4. Following collection, testicular venous blood was collected as described in 7.3.1.4. The testis was then forced through a 21G needle to disperse cells as detailed in 6.5., the resultant suspension immediately centrifuged, and the supernatant collected. In each animal, interstitial fluid was collected from the remaining testis using the drip collection technique described in 1.2.5. (see also Figure 7.1.). Peripheral venous blood was collected as described in 7.3.1.4.

8.3. RESULTS

8.3.1. Effect of anaesthesia and push-pull cannulation on testicular venous concentrations of testosterone.

The concentration of testosterone was significantly reduced under anaesthesia, as seen in Figure 8.1. Exposure of the testis for one or two hours, sampling of interstitial fluid with the push-pull cannula, or a combination of these two treatments did not have any greater effect on the levels of testosterone, than those already caused by anaesthesia. Since all these other treatments also involved anaesthesia, it is unlikely that they have in themselves lowered testosterone levels.

8.3.2. Hypophysectomy with pregnenolone supplementation

The increasing doses of pregnenolone supplementation to hypophysectomised animals resulted in an increased testosterone production. As seen in Figure 8.2., provision of 2 mg

Figure 8.1. Testicular venous blood levels of testosterone in adult rats : (IMM) immediately after induction of anaesthesia with pentobarbitone, (ANA) after one hours anaesthesia, (EXP) after exposure of the testis of an anaesthetised animal for one hour, (P/P) after one hours sampling of interstitial fluid from the testis of an anaesthetised animal with a push-pull cannula, (2EXP) after exposure of testes for 2 hours, (P/P + EXP) after one hours sampling of IF fluid with a push-pull cannula and one hours exposure of the testis. All treatments involved anaesthesia, which significantly ($P < 0.001$, t-test) lowered venous testosterone levels. However, no treatment reduced testosterone levels more than was induced by anaesthesia alone, and levels measured after some treatments remained significantly greater ($P < 0.05$) than those measured under anaesthesia alone (*). Values are mean $\pm$ standard error of the mean, n=4.

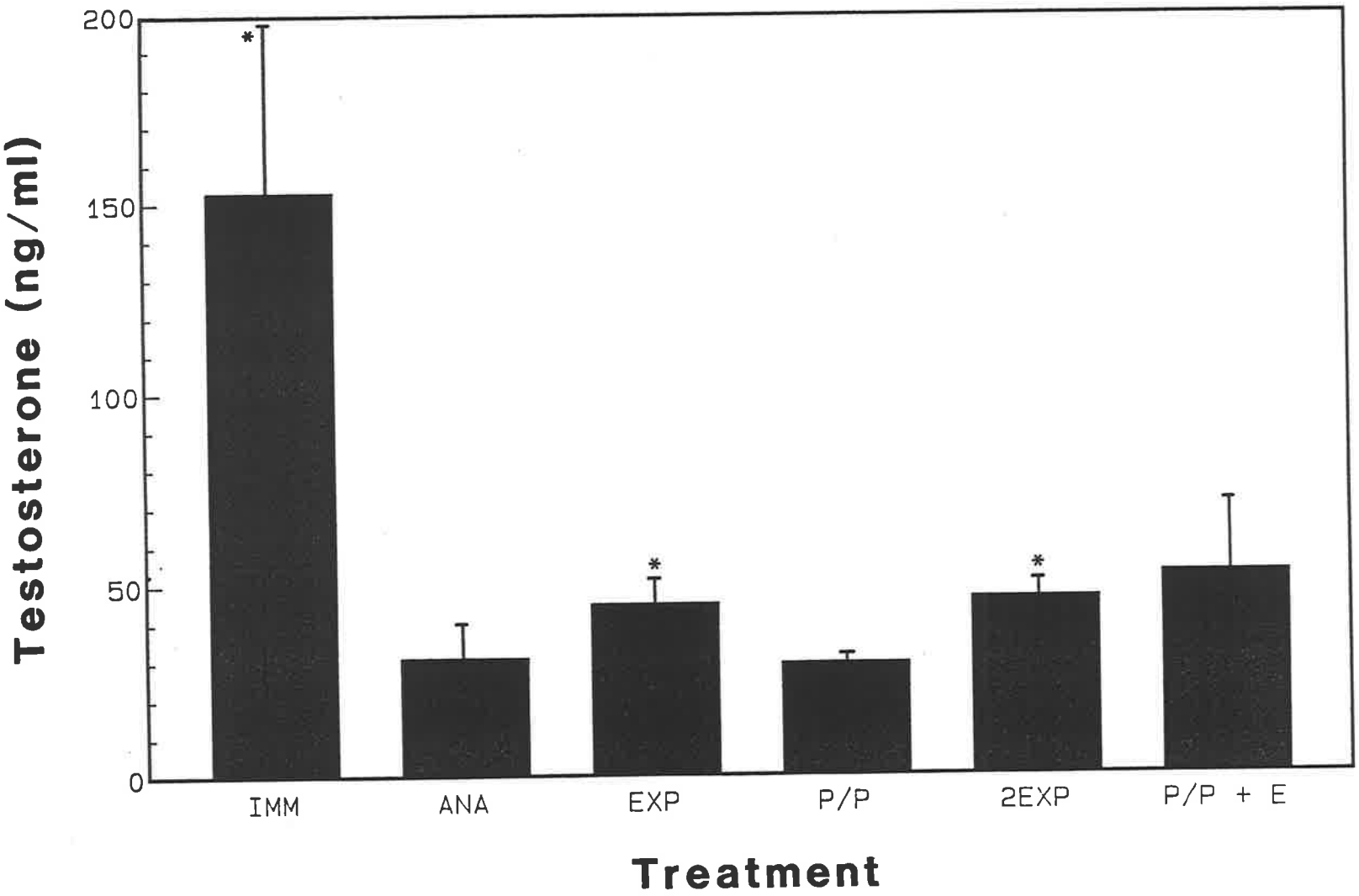
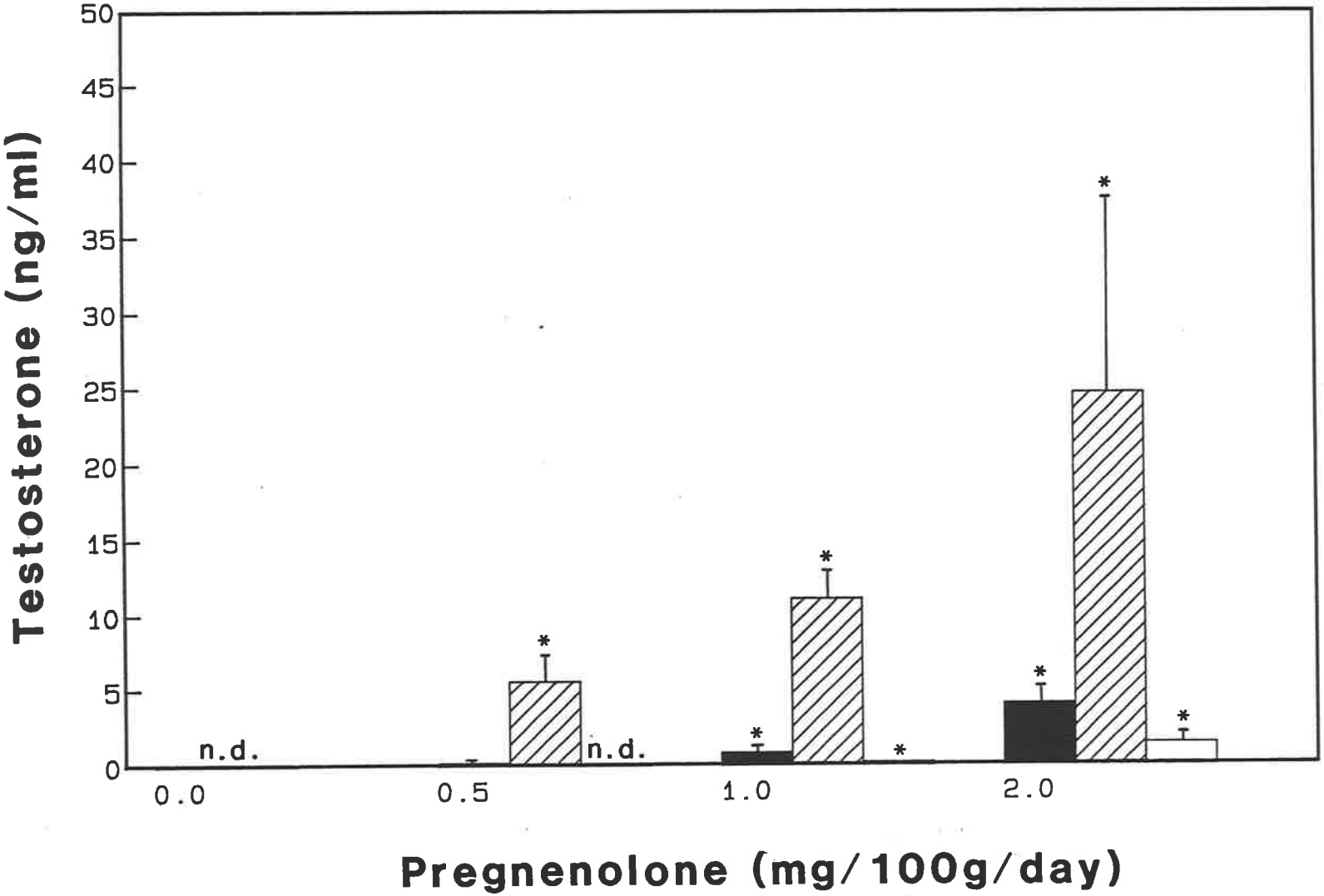

Testosterone (ng/ml)
200
150
100
50
0
*
*
*
IMM
ANA
EXP
P/P
2EXP
P/P + E
Treatment

Figure 8.2. Testosterone levels in peripheral blood plasma (open bars), testicular venous blood plasma (hatched bars) and testicular interstitial fluid (solid bars) of hypophysectomised adult rats treated with 0, 0.5, 1.0, or 2.0 mg Pregnenolone/100g bw/day for 14 days after hypophysectomy. Values are mean ± standard error of the mean, n=4. *: values significantly different ($P < 0.05$) from untreated animals.


Testosterone (ng/ml)
50
45
40
35
30
25
20
15
10
5
0
n.d.
n.d.
*
0.0
0.5
1.0
2.0
Pregnenolone (mg/100g/day)

pregenenolone/100 g bw/day was sufficient to produce levels of testosterone in testicular venous blood comparable to those levels in anaesthetised control animals (see Figure 8.1.). In all groups in which testosterone levels were detectable, interstitial fluid levels were 4-6 times lower than testicular venous blood levels, but greater than peripheral venous levels. As seen in Figure 8.3., the 2 mg dose of pregnenolone was able to maintain testis size, but accessory organs (seminal vesicles shown) have regressed to the same extent as those in vehicle treated, hypophysectomised-control animals

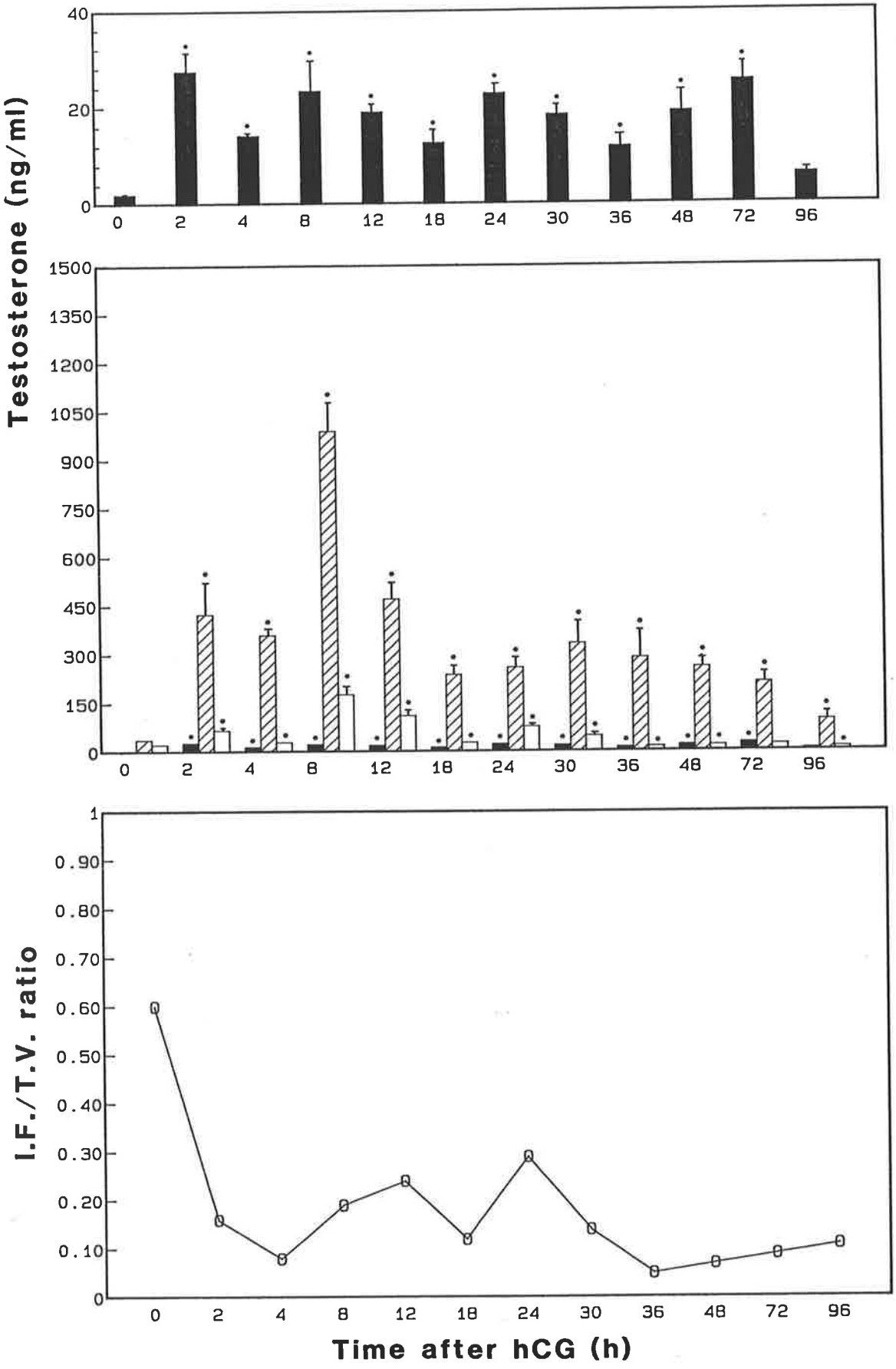
8.3.3. Human Chorionic Gonadotrophin

Following a single injection of hCG, levels of testosterone peaked in peripheral venous blood at 2, 12, and 72 hours (Figure 8.4.a). While there is a suggestion of a peak also occurring at 24 hours, this in most likely caused by low values at 18 hours giving a false "low", and splitting the peak normally found around 16 hours (see Sowerbutts et al, 1986). While both the testis vein and interstitial fluid samples show changes (Figure 8.4.b), these do not parallel the peripheral testosterone levels, particularly at 72 hours. Levels of testosterone in testicular venous blood peaked at 2 and 8 hours after hCG. Again, low values at 18 hours appear to complicate interpretation of the results. Interstitial fluid levels of testosterone showed similar trends to those observed in testicular venous blood. However these levels are again consistently lower than testicular venous blood levels. The administration of hCG in fact appears to increase the discrepancy between the interstitial fluid levels and the venous blood levels. When the ratio of testosterone in

Figure 8.3. Testes (T), Seminal Vesicles (S) and Adrenals (A) from control (C), hypophysectomised (H), and hypophysectomised - pregnenolone supplemented (P, 2mg/100g bw/day) adult male rats. Adrenal size is apparently unaffected by hypophysectomy or pregnenolone supplementation. Testis and seminal vesicle size are significantly reduced by hypophysectomy. Pregnenolone supplementation of hypophysectomised animals maintains testis size, but seminal vesicles regress to the same extent seen in untreated hypophysectomised animals.


A
S
T
C
H
P

Figure 8.4. Testosterone levels up to 96 hours after a single subcutaneous injection of 50 i.u. hCG. : (a) in peripheral blood plasma, and (b) in testicular venous blood (hatched bars) and interstitial fluid (open bars) Peripheral blood plasma levels (solid bars) are also represented in (b) for direct comparison of concentrations in the different fluids. Values presented are means $\pm$ standard error of the mean, n=4. * : values significantly different ($P < 0.05$) from untreated animals. The ratio of mean testosterone levels in interstitial fluid compared to those in testicular venous blood is shown in (c), and reflects the relative changes in concentration of testosterone in these two fluid compartments.


Testosterone (ng/ml)
40
20
0
1500
1350
1200
1050
900
750
600
450
300
150
0
I.F./T.V. ratio
1
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
0 2 4 8 12 18 24 30 36 48 72 96
Time after hCG (h)

interstitial fluid / testis vein is plotted against time (Figure 8.4.c), the ratio declines for the first 4 hours following hCG administration, indicating a greater increase in venous blood levels compared to interstitial fluid levels. From 4 to 24 hours post hCG, the value of the ratio increases again (omitting the decline at 18 hours), while testosterone levels in venous blood and interstitial fluid peak at 8 hours and then fall (Figure 8.4.b). This suggests that the changes in interstitial levels are not as great as those in venous blood. The increase in interstitial fluid testosterone from 4 to eight hours is proportionately greater than the increase in venous blood levels at this time. The ratio value continues to increase between 8 and 24 hours when testosterone levels are falling in both venous blood and interstitial fluid, and suggests that the decline in venous testosterone levels is proportionately greater than that seen in interstitial fluid. From 24 hours, the ratio declines to 36 hours, and slowly begins to rise to 96 hours post hCG.

8.3.4. Ethane Dimethane Sulphonate

Three days after administration of EDS, testosterone levels were significantly reduced and were almost undectable in interstitial fluid, testicular venous blood and peripheral venous blood (Table 8.1.). That the levels in these three fluids are not significantly different suggests that this basal level of testosterone is not of testicular origin.

Table 8.1. Levels of testosterone in posterior vena cava and testicular venous blood plasma, and testicular interstitial fluid in adult rats 3 days after treatment with DMSO/water (Control) or Ethane Dimethane Sulphonate (EDS, 75mg/kg bw in DMSO/water). Values are means ± SEM, n=4.

	Testosterone (ng/ml)		
	Testicular venous blood plasma	Interstitial fluid	Posterior Vena Cava
CONTROL	37.52 ± 6.41	22.63 ± 4.24	2.05 ± 0.34
EDS	0.329 ± 0.05	0.32 ± 1.00	0.32 ± 0.06

8.3.5. Heat treatment

Peripheral levels of testosterone were not greatly affected by heating of the testes (Figure 8.5.). Up to 28 days after heating, peripheral testosterone levels were no different to control levels. From 28 days to 63 days there was a slight, but significant ($P < 0.05$), increase in peripheral testosterone, although the level measured at 49 days after heating was not significantly different from control. Testicular venous levels were increased by 14 days post heating, and remained elevated until 35 days after heating. The levels measured in the testis vein at 49 and 63 days were significantly lower than control levels. Interstitial fluid levels of testosterone are possibly the most interesting data from this experiment. By 7 days after heating, interstitial levels of testosterone are significantly higher than control levels, and continue to increase to 35 days after heating. At this time, they are not signifcantly different from testicular venous levels, and this relationship is still found at 49 days post heating. By 63 days, interstitial levels of testosterone are again lower than testis vein levels, but are still significantly higher than control levels.

8.3.6. Efferent Duct Ligation

Results obtained after bilateral efferent duct ligation are shown in Figure 8.6. Peripheral levels of testosterone were significantly reduced at 7 and 14 days after treatment, returning to control levels by 21 days after duct ligation. Testis vein levels of testosterone were also significantly reduced by 7 days after treatment, returning

Figure 8.5. Levels of testosterone in peripheral blood plasma (open bars), testicular venous blood plasma (hatched bars), and testicular interstitial fluid (solid bars) of adult rats up to 63 days after heating of both testes at 43^{o}C for 30 minutes. Values presented are means $\pm$ standard error of the mean, n=4. * : values significantly different ($P < 0.05$) from untreated animals.

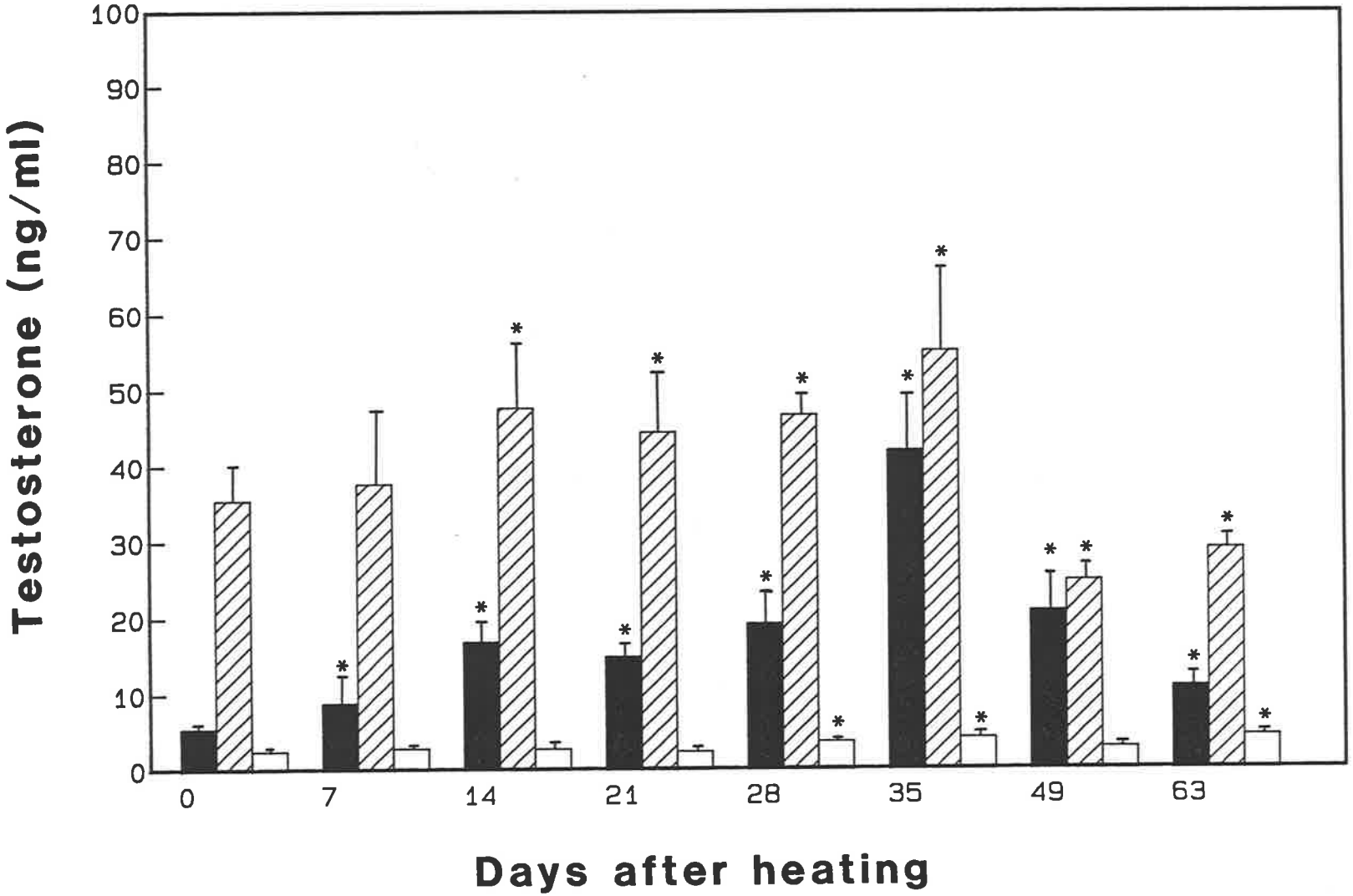
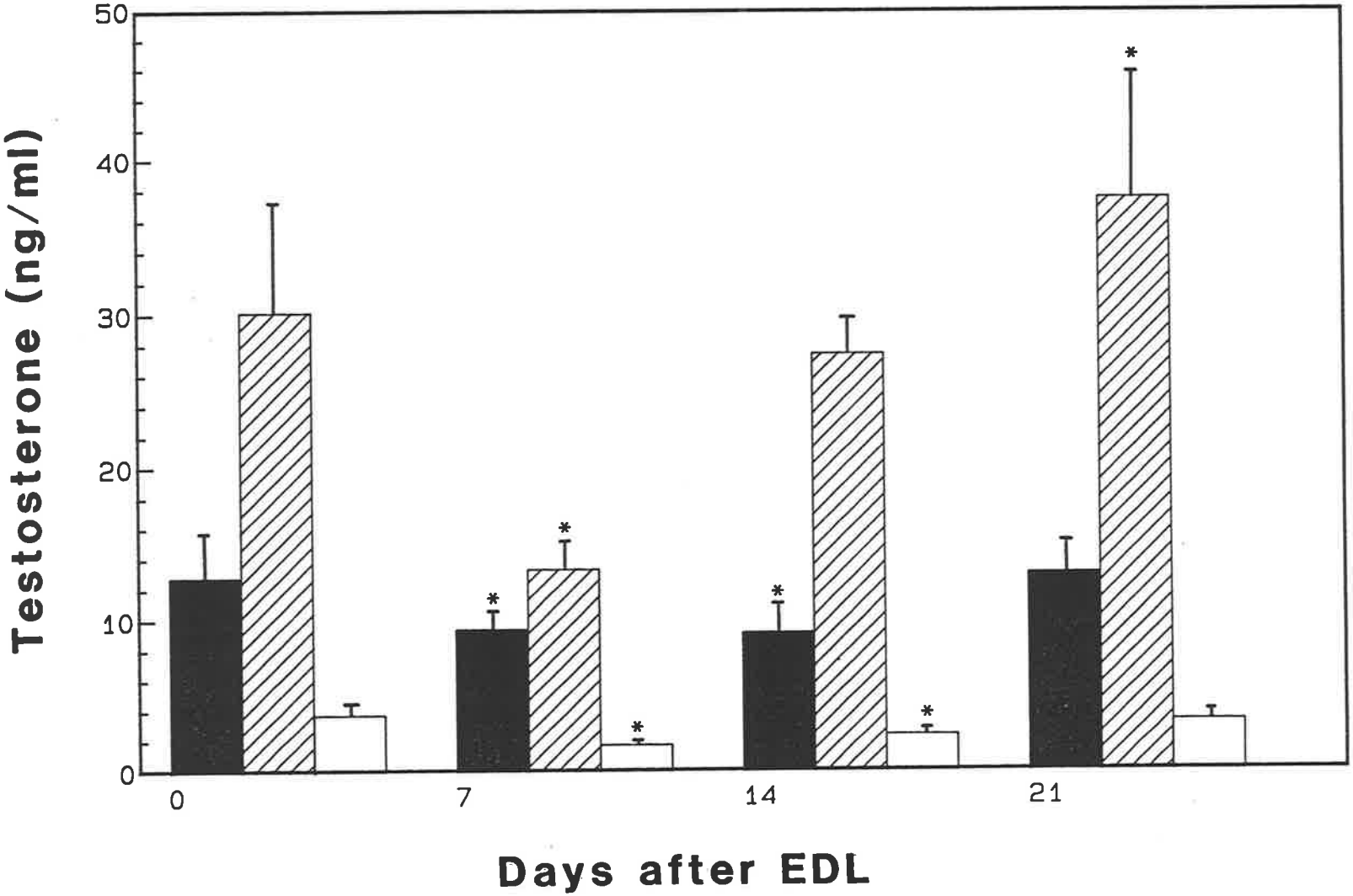

Testosterone (ng/ml)
100
90
80
70
60
50
40
30
20
10
0
0
7
14
21
28
35
49
63
Days after heating

Figure 8.6. Levels of testosterone in peripheral blood plasma (open bars), testicular venous blood plasma (hatched bars), and testicular interstitial fluid (solid bars) of adult rats at 0,7,14, and 21 days after bilateral ligation of testicular efferent ducts. Values presented are means ± standard error of the mean, n=4. * : values significantly different ($P < 0.05$) from untreated animals.


Testosterone (ng/ml)
50
40
30
20
10
0
0
7
14
21
Days after EDL
*

to control levels by 14 days after EDL. However, these levels continued to rise, and were significantly increased by 21 days after treatment. Interstitial fluid levels, were significantly lower than testis vein levels at all times. However, the profile of testosterone levels in interstitial fluid was much more like that of peripheral levels, than of testis vein concentrations. Interstitial fluid levels of testosterone were significantly reduced by 7 and 14 days after duct ligation, returning to control levels by 21 days.

8.3.7. Testosterone Implants

Testosterone-filled silastic implants raised testosterone levels in peripheral venous blood significantly within 7 days (Table 8.2.), and there was also a significant ($P < 0.01$) linear correlation between size of implant and peripheral levels of testosterone. Testicular venous levels of testosterone were significantly increased in animals with the 12cm and 16cm implants. While peripheral venous levels were significantly ($P < 0.05$) less than testicular venous levels in control animals, they were similar to testicular venous levels in animals implanted with 2cm, 4cm, 8cm, and 12cm implants, and were significantly ($P < 0.05$) greater than testicular venous levels in animals with 16cm implants. This suggests that in the control animals, the testes are the major source of testosterone, and that androgen production by the testes was significantly reduced in the presence of testosterone implants. That peripheral levels of testosterone are greater than testicular venous levels in animals with 16cm implants suggests that testicular production of androgen in these animals is less than that released from the implants, and may in fact have ceased

Table 8.2. Levels of testosterone in fluids collected from adult male rats implanted with various sizes of testosterone-filled silastic capsules for seven days. In each animal, samples of testicular interstitial fluid were collected from one testis with a push-pull cannula (I.F.) and from the contralateral testis by drip collection (Drip). Posterior vena cava (PVC) and testicular venous (TV) blood plasma were also sampled, as was the supernatant collected after dispersion of the testis sampled with the push-pull cannula (Sup). Values presented are Means ± SEM, n=4 animals per group. Analysis of variance was used to detect treatment differences, and where appropriate the Least Significant Difference value (LSD, 5%) is listed (n.s. indicates no significant difference).

Implant Length	Testosterone (ng/ml)					
(cm)	I.F.	Drip	TV	PVC	Sup	LSD
Control	9.94 ± 0.85	101.36 ± 7.78	12.63 ± 2.05	1.01 ± 0.19	20.50 ± 1.23	8.16
2	10.38 ± 2.47	14.44 ± 0.75	8.09 ± 1.14	6.75 ± 1.14	19.03 ± 2.69	4.89
4	7.94 ± 1.25	14.42 ± 0.70	9.99 ± 0.59	8.12 ± 0.98	14.71 ± 1.42	2.83
8	12.18 ± 5.02	16.84 ± 3.39	15.84 ± 2.84	18.35 ± 1.98	15.28 ± 0.89	7.69
12	13.69 ± 3.19	16.83 ± 1.14	19.77 ± 2.94	27.66 ± 5.70	16.31 ± 0.58	8.23
16	16.21 ± 0.72	22.98 ± 3.20	24.72 ± 0.81	30.03 ± 1.21	18.41 ± 0.92	3.83
LSD	n.s.	9.12	4.77	6.27	n.s.	

altogether. Drip collected interstitial fluid levels of testosterone were significantly reduced in animals with implants, and there was no significant difference in the levels of testosterone in drip-collected fluid between any implanted animals, regardless of implant size. Drip collected testosterone levels remained greater than peripheral and testicular venous blood levels with the use of 2cm and 4cm implants; were the same as peripheral and testicular venous levels with 8cm and 12cm implants, and were lower than blood levels with the use of 16cm implants. In contrast, push-pull collected fluid levels of testosterone were significantly greater than peripheral venous blood levels, but not significantly different from testicular venous blood levels in control animals. Furthermore, push-pull fluid levels were not significantly different from either peripheral or testicular venous blood levels with the use of 2cm, 4cm, 8cm or 12cm implants, and were significantly less than peripheral and testicular venous blood levels in animals with 16cm implants. Despite an apparent increase in push-pull fluid levels of testosterone with increasing implant size (Table 8.2.), levels of testosterone in push-pull fluid were not significantly different between any of the treatment groups or the control group. Use of 12cm and 16cm implants caused a significant increase in testosterone levels in testicular venous blood, but push-pull fluid levels were not affected. The levels of testosterone in drip-collected fluid from control animals were significantly ($P < 0.001$) greater than levels in push-pull collected fluid. However, in implanted animals the testosterone concentrations in these two fluids were not significantly different. The concentration of testosterone from control animals, in testicular fluid collected as supernatant from dispersed cells, was significantly

greater than that in peripheral venous plasma, but not significantly different from testicular venous plasma levels. There was no significant difference in supernatant levels of testosterone between control animals and any of the implanted animals. Supernatant levels of testosterone were significantly greater ($P < 0.05$) than push-pull collected levels in control, 2cm, and 4cm implanted animals, but were not significantly different in animals with 8cm, 12cm and 16cm implants. Drip-collected fluid levels of testosterone were significantly ($P < 0.001$) greater than supernatant levels in control animals, but not in implanted animals.

8.4. DISCUSSION

The results of these experiments have all provided support for the theory proposed in the previous chapter, that Leydig cells are likely to secrete as much testosterone into testicular venous blood as they do into interstitial fluid. The general effect of anaesthesia on lowering of blood testosterone levels is unfortunate. Setchell and Galil (1983) have also reported on the problem faced with pentobarbitone anaesthesia and the measurement of testosterone production and testicular blood flow, due to effects on gonadotrophin secretion. Ether has been reported to cause a rapid decline in testosterone production in rats (Bardin and Petersen, 1967; Fariss et al, 1969). Setchell et al (1965) have reported light pentobarbitone anaesthesia to lower testosterone secretion in rams in the days following anaesthesia, although no difference was noted at the time of induction. The effects of other anaesthetics are worthy of closer investigation, although it would obviously be useful if a method could

be developed for sampling in the unanaesthetised animal.

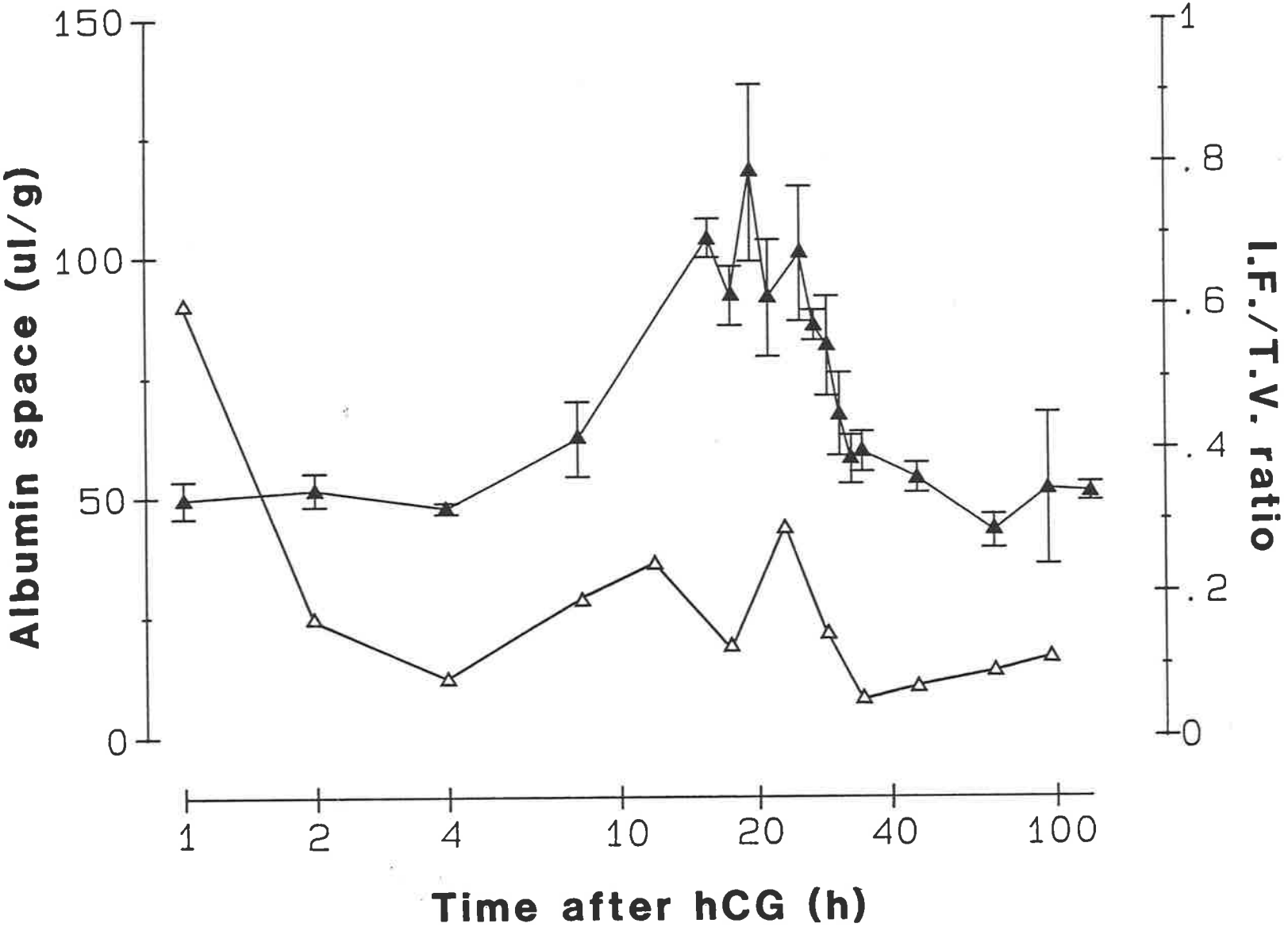
Previous reports of pregnenolone supplementation after hypophysectomy, have found intratesticular levels of testosterone to be maintained with the higher doses of pregnenolone despite low peripheral testosterone levels (Harris and Bartke, 1975; Anthony et al, 1984; Turner et al, 1985). The maximum dose of pregnenolone used by these authors was equivalent to 1 mg/100g bw/day. At this dose, testis vein levels of testosterone were lower than control values and the results of the present study agree with the previous studies in this regard. However, when the pregnenolone dose was increased to 2 mg/100g bw/day in the present study, testis vein levels of testosterone were not significantly different from normal controls. Turner et al (1985) collected testicular interstitial fluid from pregnenolone-injected hypophysectomised animals by drip collection, and found interstitial levels to be significantly greater than testicular venous blood levels, although levels in both these fluids were less than in normal control animals. In contrast to the findings of Turner et al (1985), the testosterone levels in push-pull interstitial fluid samples obtained in the present study were significantly lower than those in testicular venous samples. However, all values were less than those obtained from normal controls, which is in agreement with the report of Turner et al (1985). This again suggests that in drip collected fluid, metabolism may continue after isolation of the organ, and the resultant metabolites (eg. testosterone) accumulate in the collected fluid. As raised earlier, these elevated levels may also reflect the "stores" of testosterone, rather than actual levels in the interstitium. Testis vein levels of testosterone are apparently normal with 2mg pregnenolone/100g bw/day

despite a lower than normal concentration of testosterone in interstitial fluid which may reflect a reduced blood flow. The effect of gonadotrophins on testicular blood flow is controversial (see Daehlin et al, 1985). However, Bindon and Waites (1968) have reported testicular blood flow to be decreased after hypophysectomy in mice, and Setchell et al (1969) observed reductions in testicular blood flow in rats after hypophysectomy. Setchell and Galil (1983) have provided some evidence for the involvement of seminiferous tubules in regulating blood flow, and while pregnenolone administration to hypophysectomised animals can maintain tubular ABP concentrations and qualitatively maintain spermatogenesis (Anthony et al, 1984), other tubular factors may not be maintained by such treatment. Any gonadotrophin-dependant processes involved in regulation of interstitial hormone concentrations is likely to be affected in hypophysectomised animals, and may not be compensated for by the provision of pregnenolone.

The importance of blood flow for hormone secretion is also apparent in the results obtained after hCG administration. While both interstitial fluid and testis vein levels of testosterone showed changes after hCG (see Figure 8.4.), they did not parallel the changes in peripheral levels of testosterone. Of particular note in this regard are the values at 72 hours after hCG, when peripheral levels peaked for the third time, without concommitant peaks in interstitial or testicular venous blood. This suggests that other effects such as a change in blood flow or testosterone clearance rate contribute to the peak found in peripheral blood samples at this time. The ratio of interstitial fluid testosterone / testicular venous levels provides further evidence of the effect of vascular parameters on interstitial

fluid testosterone levels. As was noted from Figure 8.4., the peak in testis vein testosterone at 8 hours after hCG administration was followed by increasing levels of testosterone in interstitial fluid as reflected by the increasing value of the ratio up to 24 hours after hCG. This ratio data is represented in Figure 8.7., along with recently published data of Sowerbutts et al (1986) showing changes in vascular permeability after hCG (as determined by measurement of 1 hour albumin spaces). Note that the period of increasing vascular permeability which is associated with the second major peak in peripheral blood levels of testosterone, also coincides with the period of increasing testosterone levels in interstitial fluid reflected in the increasing ratio values. The data obtained from this experiment suggest that a number of factors may be involved in the responses noted after an injection of hCG, and the following hypothesis is provided as an attempt to incorporate known events with the observed responses. The initial peak in testicular venous blood probably reflects the LH-like stimulation of Leydig cells by hCG, resulting in secretion of testosterone primarily into the testicular vascular bed. From 4 to 24 hours, increasing vascular permeability combined with continued secretion by Leydig cells, results in increased levels of testosterone in interstitial fluid. The possible dynamics of this event have been previously addressed, and are likely to involve leukocytes and other inflammatory responses (see 3.5.3.1.3.). During this time, a second peak in peripheral levels occurs. Increasing levels of interstitial fluid during this time probably increase receptor occupancy by hCG. According to Sharpe (1980), testicular bound hCG increases linearly for 2 to 8 hours after injection, plateaus from 8 -16 hours and then declines towards 40

Figure 8.7. The ratio of the mean level of testosterone in testicular venous blood compared to that in testicular interstitial fluid (open triangles, represented from Figure 8.4.), and the 1-hour albumin space in the testes (solid triangles, from Sowerbutts et al, 1986) in adult male rats at various times after a single subcutaneous injection of 50 i.u. hCG. Values for albumin spaces are means ± standard error of the mean, n=4.


Albumin space (ul/g)
150
100
50
0
I.F./T.V. ratio
1
.8
.6
.4
.2
0
1
2
4
10
20
40
100
Time after hCG (h)

hours. While this might maintain Leydig cell secretion, testicular venous levels in fact are not maintained during this time. Blood flow has been reported to be increased at this time which would reduce levels of testosterone measured per unit volume. Setchell and Sharpe (1981) found testicular blood flow to be unaffected at 12 hours after hCG, but significantly increased at 16 and 20 hours after hCG, to about twice control levels. Damber et al (1981) have also reported significant increases in testicular blood flow 20-24 hours after hCG. However it is more likely that Leydig cell secretion is significantly reduced at this time. As shown in 3.5.3.1.2.1., lymph flow is increased 24 hours after an injection of hCG, and the peak in peripheral venous testosterone levels at 16 - 20 hours may be due to lymphatic clearance of interstitial fluid containing unusually high levels of testosterone at this time. After this initial 24 hours, down regulation of LH receptors on the Leydig cells has been reported (Hseuh et al, 1976; Sharpe, 1976) which may prevent further stimulation of the Leydig cells. Other factors such as testicular LHRH-like activity are also likely to impair Leydig cell function at this time (see Sharpe and Fraser, 1980). Subsequent changes after this time, are probably due to variations in testicular blood flow and testosterone clearance rates, especially for the peak in peripheral testosterone at 72 hours after hCG as already discussed.

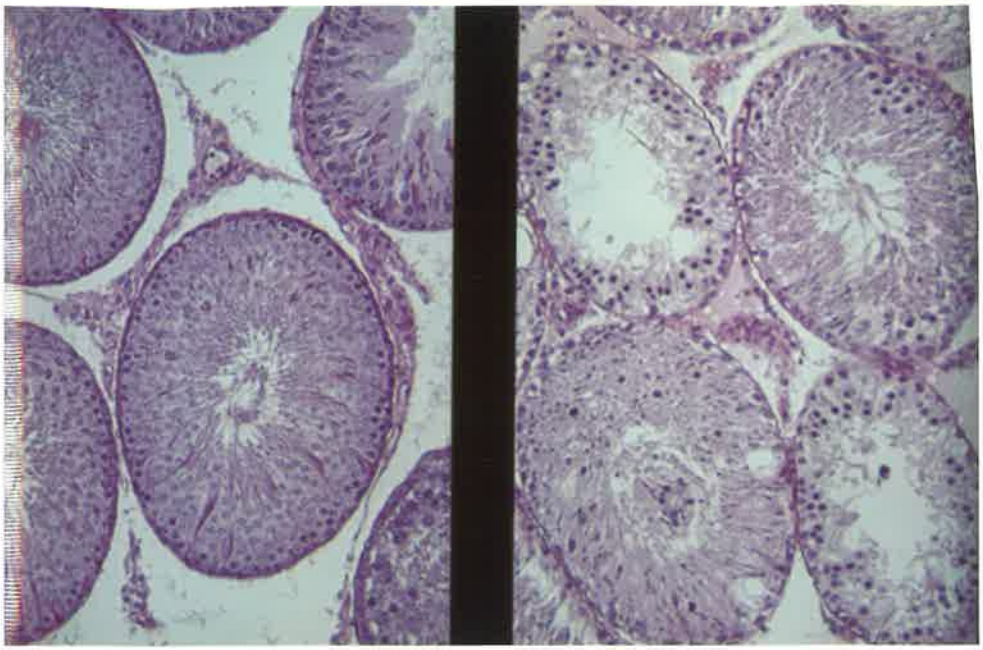
The administration of EDS reduced testosterone to almost undetectable levels 3 days after administration, which supports previous reports (Morris et al, 1986; Molenaar et al, 1985; O'Leary et al, 1986). Interstitial levels of EDS measured in fluid collected by push-pull cannulae were not significantly different to venous levels (testicular and peripheral), which suggests that these low levels are

of extra-testicular origin (probably adrenal), since Leydig cell destruction is complete by 3 days after EDS administration.

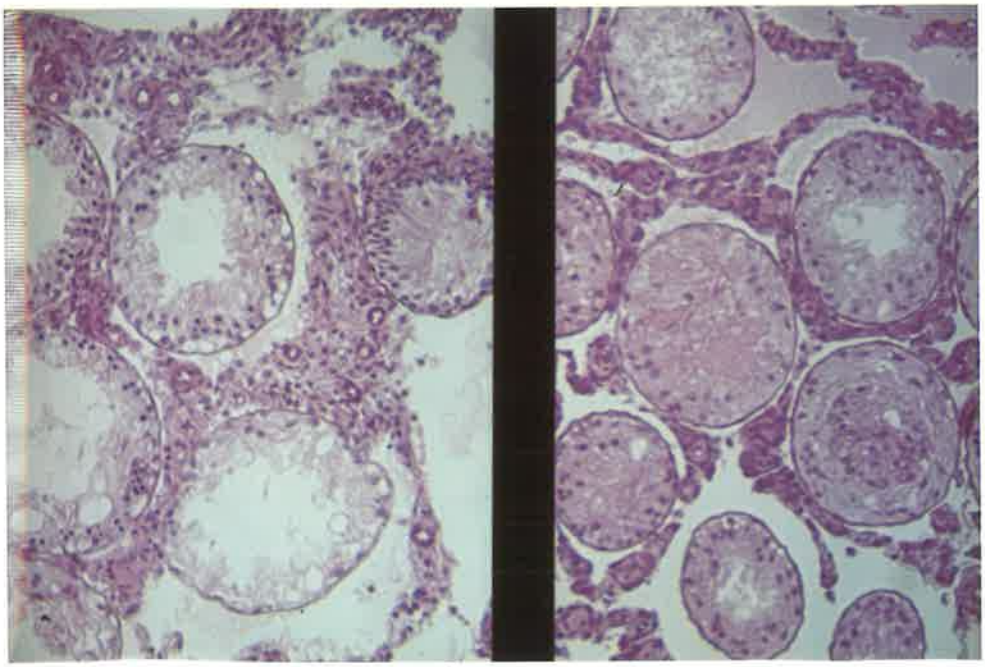
The use of the push-pull cannula to obtain interstitial fluid from testes after localised heating, has again revealed an interesting response not previously reported. The lack of consistent change in peripheral levels of testosterone has been established by other authors (see Main et al, 1978; Main and Setchell, 1980; Galil and Setchell, 1987). The testicular venous levels of testosterone were initially unaffected by heating, and then rose from 14 to 35 days after heating. Galil and Setchell (1987) reported similar findings, and correlated this rise with a decrease in testis weight. According to these authors, testis weight after heating begins to fall within 2 days, is lowest at 21 days, and partially recovers by 56 days. This weight reduction is due to disruption of spermatogenesis. The interstitial levels of testosterone determined in the present study have increased significantly as testis weight declines, and at 35 days after heat treatment are not significantly different from testicular venous levels. Setchell and Galil (1983) reported changes in blood flow during this time period, and suggested that blood flow might be regulated by tubular mass. Thus, as spermatogenesis is disrupted and tubular mass decreases, testicular blood flow would also decrease. These authors raised the problem of accounting for an inability of Leydig cells to compensate for reduced blood flow by only partially increasing testicular venous concentrations. The results of the present study suggest that greater levels of hormone in fact accumulate in interstitial fluid. It is of interest in this regard, that the peripheral levels of testosterone were also marginally, but significantly increased at these times of greater interstitial levels

(see Figure 8.5.). This may be due to lymphatic clearance of interstitial fluid testosterone. Bergh (1985) has recently described the likely paracrine regulation of Leydig cells by seminiferous tubules, albeit at specific stages of the spermatogenic cycle. It is possible that such effects might occur more generally in damaged testes, particularly during reinitiation of spermatogenesis and repopulation within the tubules. The histological presentations in Figures 8.8.a,b. show the changes in rat testes at certain times after a 30 minute exposure to 43 ^{o}C heat. The gradual disruption of spermatogenesis over time is clearly visible, and by 7 days most differentiating cells have disappeared. Late pachytene primary spermatocytes, round and elongated spermatids and the spermatozoa are absent. While a few earlier stages of primary spermatocytes persist up to 7 and 14 days, they are absent by 21 days. Of interest is the dramatic change with tubules regenerating between 28 and 35 days, and by 42 days after heating normal spermatocytes and developing spermatids are seen in the majority of tubules. By 56 days, the testis is histologically normal. That the change between 28 and 35 days should also occur at the time of maximal interstitial fluid levels of testosterone may be due to paracrine stimulation of Leydig cells by the tubules. By 49 days after heating, interstitial levels were still the same as testicular venous levels, but both were significantly lower than at 35 days, and venous levels were significantly lower than control levels. This suggests that blood flow may be increased during this stage, and in fact Setchell and Galil (1983) have reported this to be the case. By 56 days, these authors found blood flow to have returned almost to normal rates. In the present study, interstitial levels of testosterone are again reduced below testicular venous

Figure 8.8.a Histological sections from adult rat testes at various times after heating at 43°C for 30 minutes. (a) Control, (b) 7 days, (c) 14 days, and (d) 21 days following heat treatment. X 125. (from Galil, 1982; see also Figure 8.8.b).

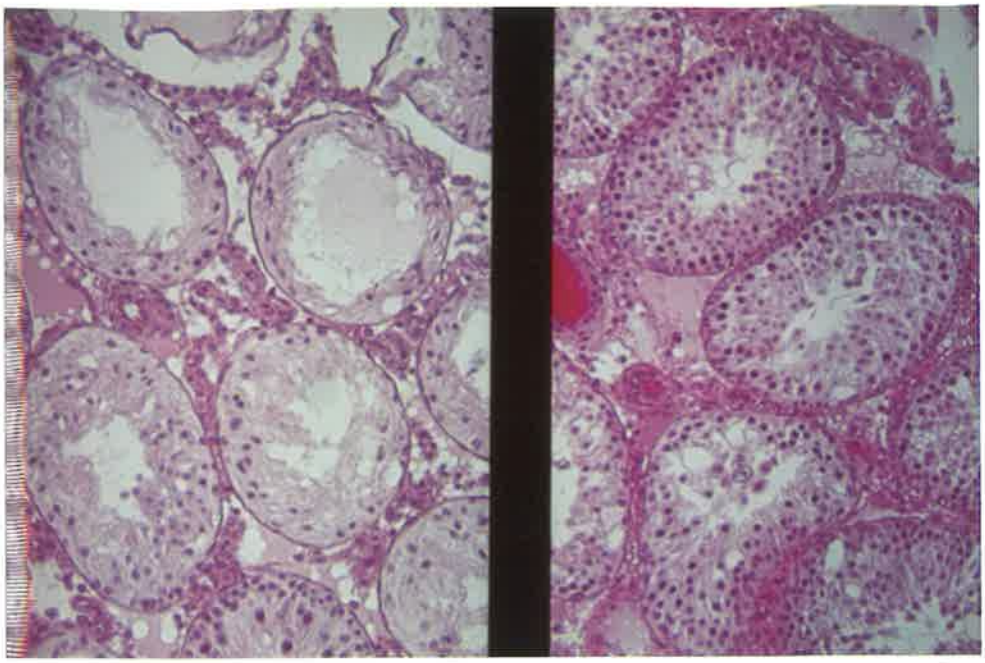


(a) (b)

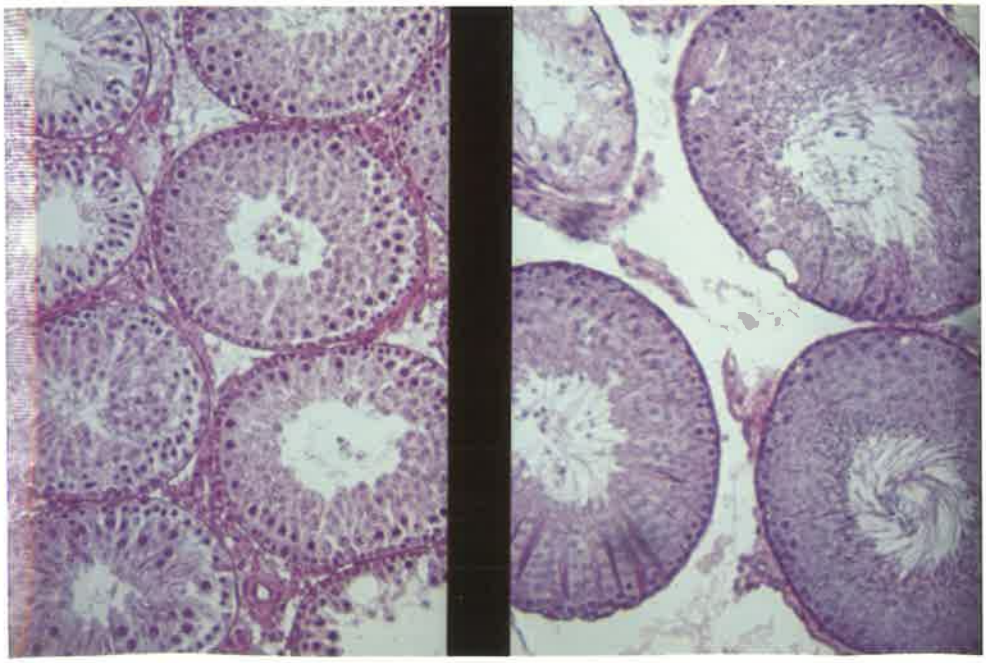


(c) (d)

Figure 8.8.b Histological sections from adult rat testes at various times after heating at 43^{o}C for 30 minutes. (e) 28 days, (f) 35 days, (g) 42 days, (h) 56 days following heat treatment. X 125. (from Galil, 1982).



e f



g h

levels by 63 days, although they are still significantly greater than control levels, and the testis vein concentrations are still significantly lower than control levels. By this time, spermatogenesis is again normal, although testis weight is still reduced. Galil (1982) reports that some tubules failed to regenerate by 42 days after heating, and contained only Sertoli cells, spermatogonia and spermatocytes. This may account for the continued differences in testosterone levels at 63 days, compared to control levels.

Ligation of the efferent ducts of the testis causes fluid secreted by the testis to be retained inside the seminiferous tubules and rete testis, resulting in enlargement and eventual degeneration. Setchell et al (1977) and Main et al (1978) have reported that serum testosterone levels are not consistently affected by EDL. Risbridger et al (1981) found serum testosterone levels to be similar to control levels at 2 weeks after EDL, but significantly reduced by 4 weeks. Main and Setchell (1980) found no difference in testicular venous levels of testosterone between bilaterally ligated animals and control animals at 21 days after ligation, but did find significant differences between unilaterally ligated and control animals at this time. These results suggest that great fluctuations in secretion of testosterone occur after bilateral ligation and comparisons of data from different authors need to be made at similar time points. The effects of bilateral ligation on gonadotrophin responses probably makes comparison of unilateral and bilateral data of questionable value. The results of the present study show minor variations in peripheral levels of testosterone which were slightly reduced at 7 and 14 days after duct ligation, but not significantly different from control levels by 21 days. This is not dissimilar to previous reports.

Of interest is the significant reduction in testicular venous levels at 7 days after ligation, which return to normal by 14 days , and are then significantly increased by 21 days. Because interstitial fluid levels do not show a similar response, it is unlikely that the venous levels are directly attributable to changes in Leydig cell secretion. It is more likely that alterations in blood flow are of consequence in this regard. Indeed, Wang et al (1983) concluded that during aspermatogenesis following irradiation (as with heat and efferent duct ligation) the capacity of the testis to secrete testosterone is severely limited by decreased testicular blood flow, not by the ability of the Leydig cells to release testosterone into their immediate environment. Wang et al (1985) have also reported blood flow to play a role in changes of testosterone secretion seen after unilateral ligation. Efferent duct ligation and heat seem to induce very similar changes in the testis, and it is not surprising that responses similar to those found after EDL were also noted in heat treated animals in the present study. Heat-treated animals did not show the reduction in testicular venous levels of testosterone at 7 days after treatment, which may relate to a difference in the aetiology of disruption in spermatogenesis. However in other respects, the changes in testosterone levels appear very similar, further highlighting the likely importance of tubular-interstitial interactions.

Much of the data on venous levels of testosterone affected by the treatments used in this study, supports existing literature. New information or ideas have been generated largely from the data collected with the push-pull cannula. This data has consistently

supported the hypothesis raised earlier regarding partitioning of testosterone levels between venous blood and interstitial fluid. Further support comes from the final experiment with the use of testosterone implants. This experiment was designed to compare the drip-collection system and the push-pull system directly. The initial discussion on the drip-collection method raised the likely problem of continuing metabolism after isolation of the organ. Deprived of blood flow, the various products of the testis would have to end up in the interstitial fluid - a factor most likely to contribute to artificially high levels of substances measured in such fluid. A number of authors have demonstrated that testicular production of testosterone is inhibited in normal animals with exogenous administration of supranormal levels of this hormone by injection or subcutaneous implant (see Cunningham and Huckins, 1979; Rea et al, 1986). If continued testicular production of testosterone is contributing to high levels in drip-collected fluid from normal animals, levels in drip-collected fluid from testosterone-supplemented animals should be dramatically reduced since intratesticular metabolism will be suppressed. The results of the present study suggest that this is indeed what happens. As soon as exogenous testosterone was provided to animals, drip collected fluid levels fell substantially. In a recent abstract, Sun et al (1986) reported the use of testosterone implants for 50 days, and found the same result, namely that drip collected testosterone levels were significantly reduced in implanted animals. In planning this experiment, it was envisaged that testosterone levels in drip collected fluid and push-pull collected fluid would be very similar in implanted animals; particularly when the implants were large enough to suppress

intratesticular production/secretion of testosterone. Again, as seen in Table 8.2., the levels measured in fluids collected by these techniques were not significantly different in implanted animals. This is interpreted as support for the theory behind the use of the push-pull cannula, and provides further evidence that the high levels of testosterone found in drip-collected fluid from unimplanted animals are artifactual due to problems with the technique.

It is interesting that the testosterone levels in supernatant fluid from cell-dispersed testes are not significantly different from the drip collected fluid levels in implanted animals. This supernatant fluid contains both interstitial fluid and tubular fluid in roughly equal proportions, although interstitial fluid probably comprises a greater proportion in aspermatogenic testes (Galil and Setchell, 1987). Comhaire and Vermeulen (1976) and Davies et al (1979) reported similar concentrations of testosterone in tubular and interstitial fluid and the results in the present study provide further evidence for this. In 1978, Setchell et al reported a specific, saturable carrier-mechanism, probably involving facilitated diffusion, to be involved in the transport of testosterone into seminiferous tubule fluid and rete testis fluid. This study was based on comparisons of fluid / plasma ratios of labelled testosterone, 5α-dihydrotestosterone and 5α-androstan-3α,17β-diol and the lipid distribution coefficients of these steroids (see also Setchell, 1980). Fluid / plasma ratios of labelled testosterone were reduced with increased levels of cold testosterone in plasma, and the authors concluded that entry into tubular fluid was by a saturable carrier mechanism. Since entry was always down a concentration gradient, the process was thought to involve facilitated diffusion. Although these studies did not monitor

interstitial fluid, the authors considered this mechanism to be probably located in the seminiferous tubules. Androgen binding protein did not appear to be involved. The results of the present study suggest that tubular and interstitial fluid levels of testosterone may not be greatly different, and the "gradient" in fact appears to occur between the vascular and interstitial compartments (especially with the large exogenous administration of testosterone from 16cm implants). It appears that a saturable carrier mechanism does exist, but that it may in fact lie in the capillary endothelium. This is suggested because interstitial fluid and cell dispersed fluid levels plateau in implanted animals, while testicular venous and peripheral blood levels continue to rise. It is also possible that two carrier systems operate, with one transporting testosterone into interstitial fluid, and the other involved in transport of testosterone into the tubules. Testosterone administration by implants or injection to normal animals reduces gonadotrophin secretion (Buhl et al, 1982; Rea et al, 1986), and if one or other of these carrier mechanisms was regulated by gonadotrophins, it might explain some of the differences noted between the present study and the earlier studies of Setchell et al (1978). More investigations are obviously required, but this is certainly a fascinating idea. That testosterone levels in supernatant fluid from control animals are higher than testicular venous blood levels in the present study, may be due to cell damage during dispersion. The contribution of testosterone from damaged cells would not be such a problem in implanted animals when testicular hormone production was suppressed by the implants.

CHAPTER 9: INTERSTITIAL FLUID IN RAMS AND BOARS - USE OF THE PUSH-PULL CANNULA

9.1. INTRODUCTION

The investigations in the rat with the push-pull cannula suggested that the technique was more than adequate in providing access to fluid in the interstitial region under physiological conditions. Given that drip collection does not work effectively with other species (J. Closset, Universtiy of Liege, Belgium - personal communication) it was of interest to extend the use of the push-pull cannula to other species. Because of the interest in the immune status of the ram testis, this species was an obvious choice, and two animals were available for push-pull cannulation. At this time, two boars were also available for study and were included because of the completely different interstitial anatomy of the boar testis (see chapter 1). In addition to push-pull samples of interstitial fluid, blood samples were taken from a peripheral vein and testicular veins, and testicular lymph samples were also collected. In the boars, testicular venous blood was collected from an internal spermatic vein in the spermatic cord.

9.2. MATERIALS AND METHODS

Most materials and methods have been previously described in chapters 2 or 6. The apparatus required for push-pull cannulation had to be modified somewhat to allow for the different sizes of the animals. A large perspex stage was constructed to hold the testes during cannulation. The stage and micromanipulators were attatched to a large metal frame that was mounted on the operating table across the rear of the animal. This frame was positioned so that the testes could

be placed on the stage but breathing movements of the animals could not move the stage or micromanipulators. This was important because the breathing movements of the animals during anaesthesia continually moved the testes in relation to the cannulae if the testes rested on the animal.

9.2.1. Rams

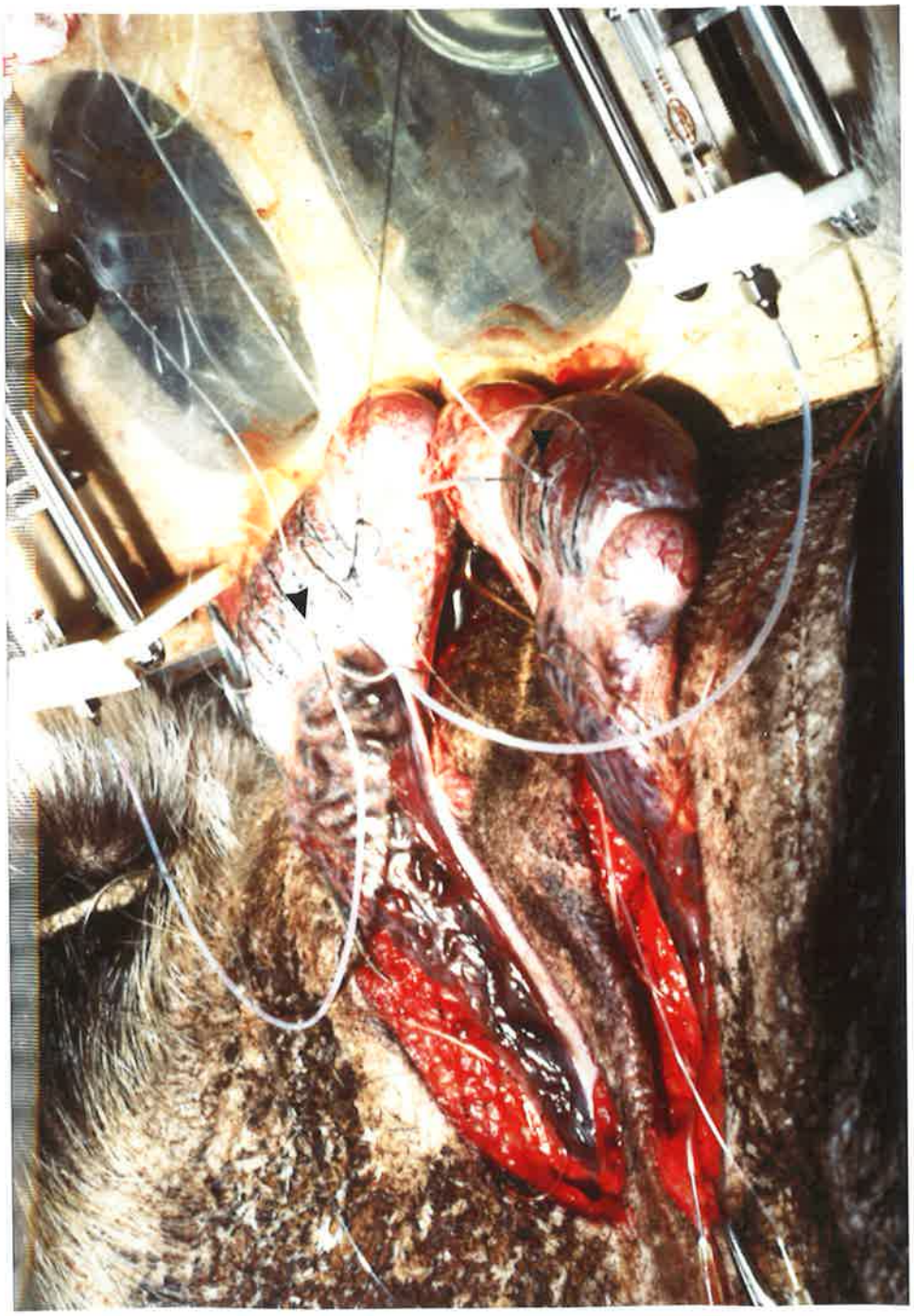
The night before surgery, each ram was given an intravenous injection of 100 uCi TOH and 90 uCi ^{125}I-human serum albumin. Rams were kept overnight without food or water, in metabolism crates in order to collect any radioactive waste. Two hours prior to surgery, another intravenous injection of 100 uCi ^{22}NaCl was administered. Rams were anaesthetised with sodium pentobarbitone as described in 2.3.1. Testes were isolated through a scrotal incision, and catheters inserted into lymphatics in the spermatic cord as described in 2.3.6. Testicular veins were exposed by resecting the head of the epididymis. Using a 26G needle, an entry hole was made into a straight vein, and a polyethylene cannula (0.2 mm I.D.,0.5 mm O.D.) containing heparinised saline, was inserted for a distance of 3 - 5 cm. Once all catheters were inserted and checked for sampling, push-pull cannulation was performed in much the same way described in 7.3.1.4. for the rat. Greater use of pointed forceps to aid entry of the cannula was necessary due to the thicker capsule of the testis. Push-pull sampling was performed simultaneously on both testes in each animal for one hour. Blood and lymph samples were collected every 15 minutes. When collections were concluded, pieces of testis where cut from the site of insertion of the push-pull cannula, placed in Bouins fixative, and

processed for histological examination. Animals were then euthenased.

9.2.2. Boars

In the experiments with the boars, it was necessary to anaesthetise the animals prior to administration of isotopes. The neuroleptic Stresnal (Azaperone, Janssen Pharmaceutica, Beerse, Belgium, 2ml/20 kg) was given by intramuscular injection to subdue the animal. An ear was then shaved, and warm water used to dilate the veins. Sodium pentobarbitone (Nembutal, Abbot, 60mg/ml) was injected into a vein to induce anaesthesia. An ear vein was then surgically exposed and cannulated with a polyvinyl catheter (0.8 mm I.D.,1.2 mm O.D.) using a method similar to that described for cannulation of the lateral saphenic vein in 2.3.1. 100 uCi of ^{125}I-human serum albumin and 100 uCi ^{22}NaCl were injected through the ear vein cannula, and two hours equilibration allowed. Testes were then exposed and cannulae inserted into lymphatics and veins in the spermatic cords and into internal spermatic veins as described for sheep in 2.3.5. Push-pull cannulation and blood and lymph sampling was performed as described in 9.2.1. Micromanipulators were unable to be used to position cannulae in boars. The tunica was so thick and tough that each time the cannulae were pushed through the entry hole, the plasticine holding the cannulae onto the microanipulators parted from the micromanipulators. So once an entry hole was made, cannulae were carefully positioned by hand. Figure 9.1. shows a boar with push-pull cannulae in position. Tissue samples from the site of insertion of the push-pull cannula were placed in Bouins fixative and processed for histological examination. Animals were then euthenased.

Figure 9.1. Photograph showing push-pull cannulae (arrow) positioned in the testes of an adult boar.



9.3. RESULTS

Due to the limited number of animals available, testosterone levels were the only measurements made in this study. The values obtained for each animal are shown in Table 9.1. Values presented for push-pull samples have been corrected for dilution as monitored by $^{22}NaCl$. Testosterone infusions have not been performed in these species, and thus final dilutions can not be accurately determined. However values corrected for the ratio of testosterone to sodium dilutions as determined in rats, are presented for comparison. In both rams and boars, testosterone levels in interstitial fluid were similar to testicular venous blood levels. Lymphatic levels of testosterone were also comparable to testicular venous blood levels in both species, but in the boar, were higher than internal spermatic venous levels.

Histology of the ram and boar testis after push-pull cannulation is presented in Figures 9.2. and 9.3. These two species have greater amounts of interstitial connective tissue than the rat, and tissue damage at the point of insertion is more obvious in these two species (compare with rat histology in Figure 7.8.). However the damage appears very localised and does not seem to be sufficient to affect hormone levels in the collected fluid.

9.4. DISCUSSION

The findings in these experiments confirm previous reports of testosterone levels in testicular fluids in rams and boars. Lindner (1963) investigated the partition of androgen between lymph and venous

Table 9.1. Levels of testosterone (ng/ml) in peripheral and testicular venous blood, testicular lymph and testicular interstitial fluid samples from adult rams and boars. Individual values, and mean values ± SEM for each species, are presented.

Sample	Ram No.1.	Ram No.2.	Ram Means	Boar No.1.	Boar No.2.	Boar Means
Blood						
Peripheral	2.0	2.21	2.11 ± 0.11	1.57	2.59	2.08 ± 0.51
Testis Vein	30.1	26.54	28.32 ± 1.77	35.06	47.77	41.42 ± 6.35
Spermatic Vein	-	-	-	21.41	22.60	22.01 ± 0.59
Testicular Lymph	31.8	23.88	27.84 ± 3.96	26.62	44.71	35.67 ± 9.04
Interstitial Fluid*	23.83	17.43	20.63 ± 3.19	15.60	16.05	15.83 ± 0.22
Corrected I.F.+	28.60	20.92	24.76 ± 3.84	18.72	19.26	18.99 ± 0.26

* : values corrected for dilution during collection as measured by dilution of radioactive sodium chloride (see main text for details).
+ : corrected for the ratio of testosterone to sodium chloride dilutions as determined in rats (see main text for details).

Figure 9.2. Histological sections of the testis of a ram after push pull cannulation: (a) this section shows the normal structure of the ram testis, which does not appear to have been affected by perfusion sampling with the push-pull cannula; (b) section from site of insertion of push-pull cannula. Most tubules appear intact, and damage from cannulation is very localised. X 80.

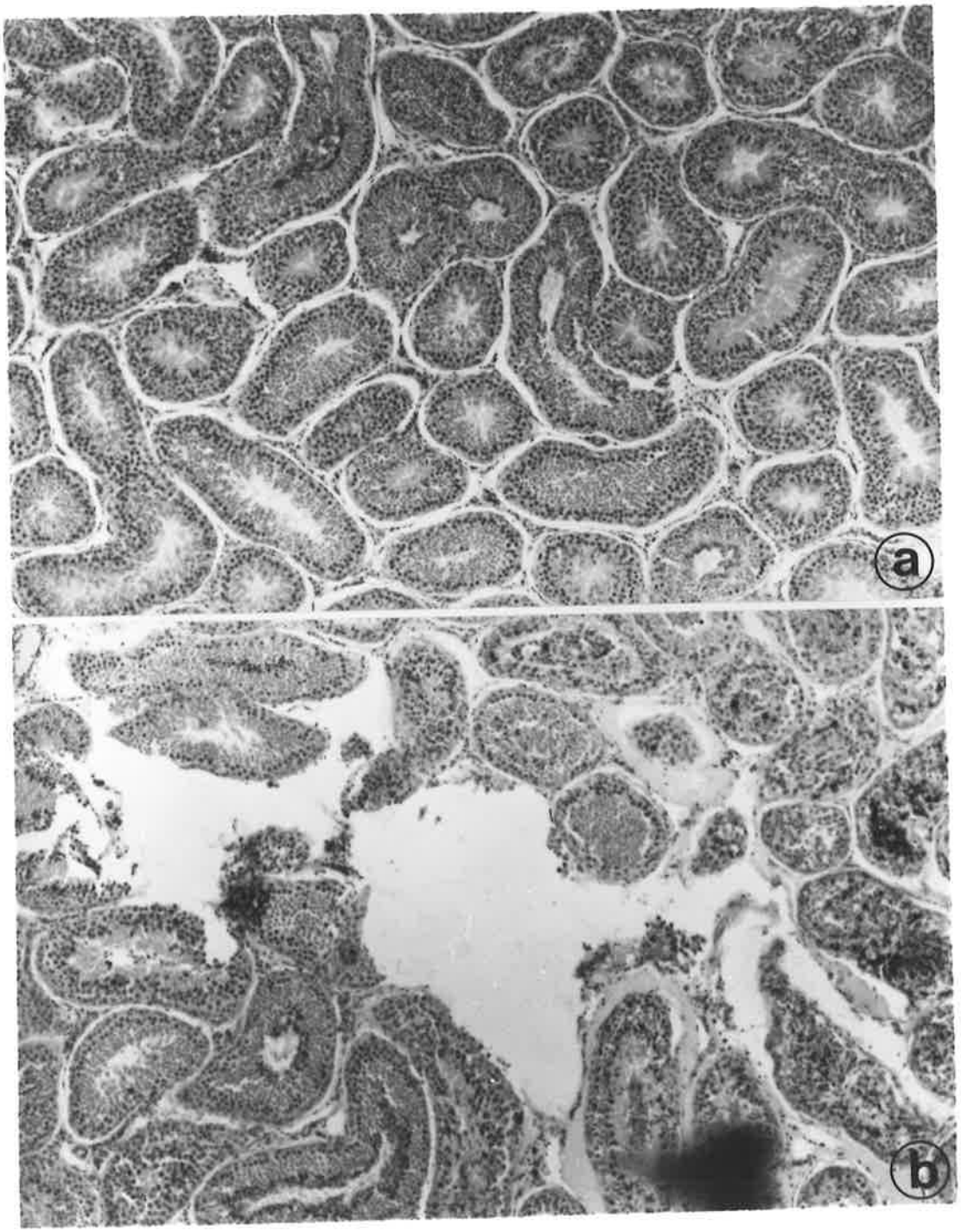
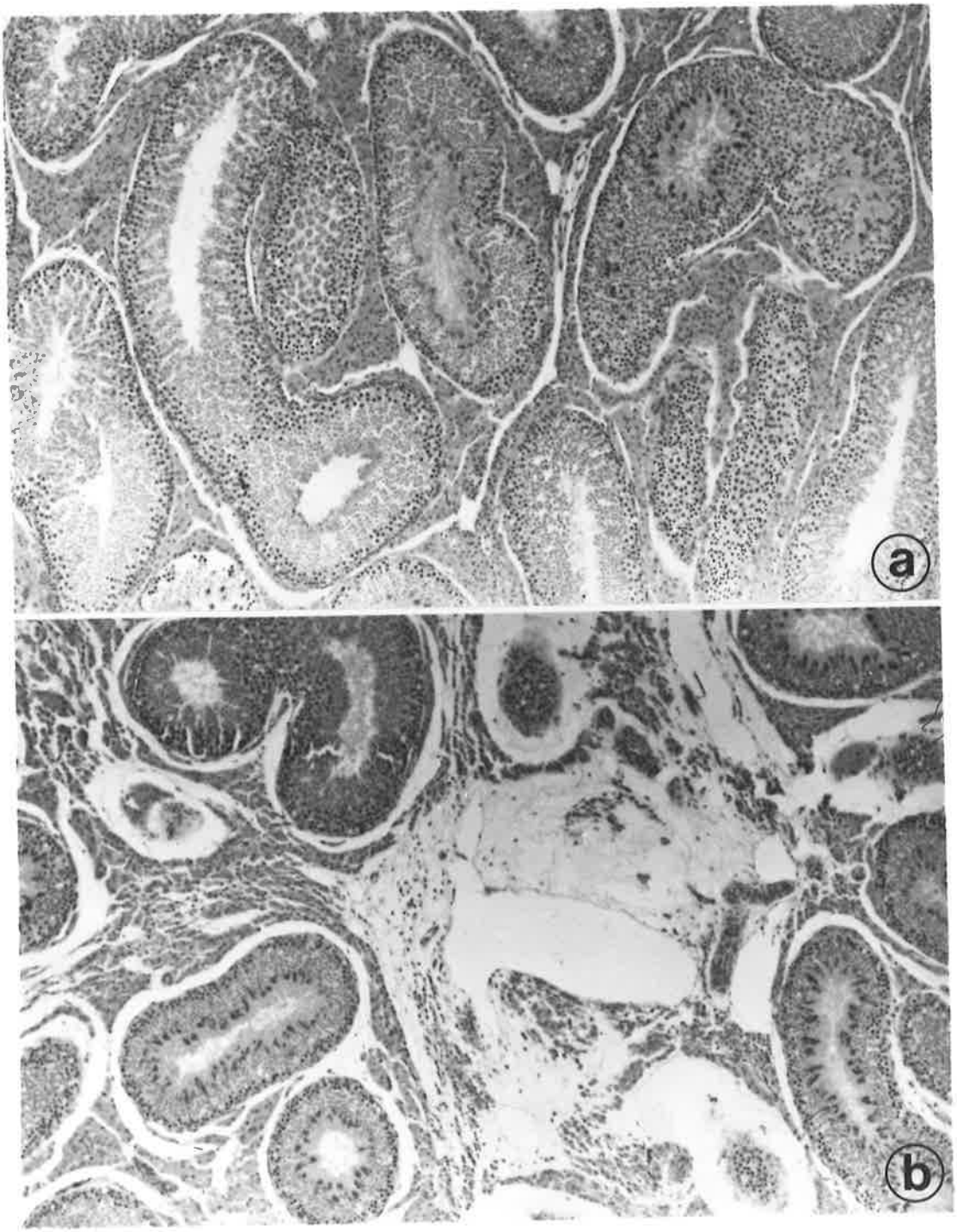

a
b

Figure 9.3. Histological sections of the testis of a boar after push pull cannulation: (a) this section shows the normal structure of the boar testis, which does not appear to have been affected by perfusion sampling with the push-pull cannula; (b) section from site of insertion of push-pull cannula. Most tubules appear intact, and damage from cannulation is very localised. X 80.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blood in the testes of rams and found testosterone levels in lymph to be lower than the levels in plasma of spermatic vein blood (ratio 0.61). Setchell et al (1983) studied levels of steroids in boar testis lymph and venous blood, and found levels of testosterone in lymph to be some twofold higher than levels in spermatic veins. This finding was seen in one pig in the present study, but not in the second animal. It is of interest that levels in the internal spermatic veins of both pigs were significantly lower than levels in testicular venous blood taken from a vein on the surface of the testis. Free and Jaffe (1975) have reported the transfer of testosterone from spermatic venous blood into testicular arterial blood in the rat, and Jacks and Setchell (1973) have confirmed this in the ram. Internal spermatic vein cannulae are inserted well up the cord, and would likely sample blood from a number of veins, after transfer had occured. The results of the present study suggest that venous-arterial transfer may also occur in the boar. Until recently, it was assumed that such transfer occurred by passive diffusion (Setchell, 1978), although Free et al (1976) did suggest that blood might pass between non-metabolic channels between artery and plexus. Anatomical evidence has been found for direct arterio-venous connections between testicular artery and pampiniform plexus or branches of the epididymal arteries and the pampiniform plexus, in the spermatic cord of the ram, bull and boar (Wensing and Dijkstra, 1981; Wensing et al, 1981; Noorduizen-Stassen, 1984).

The small number of animals available for study limits the general interpretations of the data. Likewise, it is necessary to calibrate labelled-testosterone transfer into interstitial fluid in these species before further studies are undertaken. However, the

results are encouraging, and suggest that levels of testosterone in the interstitial fluid in rams and boars are also comparable to the levels in testicular venous blood. The results also suggest that the push-pull cannula is likely to be of use in studying the interstitial fluid of a number of species, and is not limited to use in rodents.

CHAPTER 10 : IMMUNOCOMPETENT CELLS IN THE TESTES OF RATS AND RAMS

10.1. INTRODUCTION

The factors most likely to contribute to the immune privileged status of the rodent testis were reviewed in the introduction to this thesis (chapter 1), and most interest appears to focus on endogenous immunosuppressive agents such as steroids (Roubinian et al, 1977; Whitmore et al, 1985; Head and Billingham, 1985). The work in this thesis has demonstrated that the ovine testis, in contrast to the rodent testis, is not an immunologically privileged site. Since both animals have effective lymphatic drainage from the testis (rat - Tilney, 1971; McCullough, 1975; ram - Lindner, 1963; Morris and McIntosh, 1971) this suggests that some important difference exists in the interstitial region of the testis in these two species. Results with the push-pull cannula have not found any significant difference in testosterone levels between these species, and while the data is not completely conclusive, no other reports are known to detail large differences in the steroid profiles of adult male rats and rams. Elimination of Leydig cells in the rat testis with EDS (chapter 3) effectively removed the immunological protection from the testis, and grafts were rapidly rejected. However, the ram testis also contains Leydig cells, and this suggests that some other factor must be implicated in this result.

Macrophages have been identified in the interstitial tissue of the rat testis (Christensen and Gillum, 1969; Clark, 1975; Niemi et al, 1986), and have been shown to contain class II major histocompatability antigen (Niemi et al, 1986) and immunoglobulin (Fc) receptors on their surface (Miller et al, 1983). The presence and composition of lymphocyte populations have not been systematically

investigated, although some reports make reference to their presence in the testicular interstitium (Ritchie et al, 1984; Niemi et al, 1986). Macrophages act in general by removing and digesting excess antigen, and their role in antigen presentation for the immune response has been discussed in chapter 1. However, if antigen interacts indiscriminaetly with lymphocytes in a location unfavourable for cell co-operation, tolerance is induced instead of immunity (Fawcett, 1986).

The study to be reported in this section was undertaken to examine the presence of macrophages and the various classes of lymphocytes in the ram and the rat testis, in order to investigate possible differences in the immune-cellular compostition of the interstitial tissue compartments of these two species which might account for the different response to tissue allografts.

10.2. EXPERIMENTAL PROCEDURES

The experiments to be described were initiated during a visit to the Department of Anatomy, Institute of Biomedicine, University of Turku in Finland. Much of the work was performed by Dr Passi Pollanen and Professor Niemi after my return to Australia. I am most grateful for their efforts on my behalf, and for their permission to include the work in this thesis.

10.2.1. Animals

Sprague-Dawley rats and Finnish Land-race sheep were used in these studies.

10.2.2. Cytochemistry

Macrophages were identified using histochemical demonstration of non-specific acid-phosphatase activity (Cohn and Weiner, 1963).

10.2.2.1. Preparation of Tissue

Rat testes and pieces of testes from slaughtered rams were frozen in liquid nitrogen and 5 um sections were cut in a cryostat. Sections were dried at room temperature and fixed in cold acetone (-20 ^{o}C) for 15 minutes.

10.2.2.2. Acid-phosphatase detection

Acid phosphatase activity was demonstrated with the lead salt method of Gomori (1941), and the azo-dye coupling method of Barka and Anderson (1962).

10.2.2.2.1. Gomori method

10.2.2.2.1.1. Incubation medium (pH 5.0)

0.5 M veronal acetate buffer, pH 5.0	10 ml.
Sodium B-glycerophosphate	32 mg.
Lead Nitrate	20 mg.

Lead nitrate must be completely dissolved in the

buffer before adding the sodium B-glycerophosphate.

10.2.2.2.1.2. Procedure

1. Sections were placed in incubating medium at 37 °C for 30 minutes to 2 hours.
2. Sections were washed well in distilled water.
3. 1% ammonium sulphide (freshly prepared) was added to sections for 2 minutes.
4. Sections were washed again in distilled water.
5. Counterstaining was performed with 2% Methyl green (chloroform extracted) for 5 minutes.
6. Sections were washed in tap water.
7. Dehydration in alcohol and clearing of sections in xylene was carried out prior to mounting in DPX.

10.2.2.2.2. Azo-dye coupling method

10.2.2.2.2.1. Incubation Medium

Stock Solutions (stored at 4 °C)

Solution A : Pararosanilin

Pararosanilin HCl	2 gm
2 N HCl	50 ml

Heat gently to boiling point, cool and filter.

Solution B : Sodium Nitrite

$NaNO_2$	400 mg
Distilled water	10 ml

Make fresh every four days.

Solution C : Veronal Acetate Stock

Sodium acetate ($CH_3COONa.3H_2O$)	1.95 gm
Barbitone sodium	2.95 gm
Distilled water	100 ml

Solution D : Substrate

Napthol ASBl phosphate (Sigma)	50 mg
Dimethyl Formamide	5 ml

Medium

1. Solution A 0.4 ml
   Solution B 0.4 ml

   Wait one minute

2. Add
   Solution C 2.5 ml
   Solution D 0.5 ml
   Distilled Water 6.0 ml

   Adjust pH to 4.7 using 1 N NaOH and filter.

10.2.2.2.2.2. Procedure

1. Sections were placed in incubating medium at 37 ^{o}C for 10 - 60 minutes.
2. Sections were then washed well in distilled water.
3. Nuclei were counterstained with 2% methyl green for 5 minutes.
4. Sections washed briefly in tap water.
5. Sections were then dehydrated in alcohol, cleared in

xylene and mounted in DPX.

10.2.3. Immunocytochemistry

Specific monoclonal antibodies were used to examine lymphocyte populations and the localisation of major histocompatability complex antigens in testes from rats and rams.

10.2.3.1. Preparation of tissue

Small pieces of fresh tissue were fixed by immersion in phosphate-buffered 4% p-formaldehyde overnight. Tissues were kept in an arabicum-sucrose medium until sectioned in a cryostat.

10.2.3.2. Detection of Cell type-specific antigens

Indirect immunoperoxidase staining was used to visualise specific antigens. Antibodies used are detailed in Table 10.1.

Procedure

1. Frozen sections were rehydrated by incubating slides in PBS for 3 - 5 minutes.
2. Non-specific binding sites were blocked by incubation of sections in 5% normal rabbit serum.
3. Primary antibody was applied to sections, which were then incubated for 60 minutes at room temperature. Control sections were incubated with normal mouse

Table 10.1. The antibodies used to identify various subtypes of lymphocytes and the MHC II antigen-positive cells in the rat and the ram testis.

Antigen	Specificity	Reference
MRC Ox 17*	rat MHC II antigen	Mayrhofer et al, 1983
MRC Ox 19*	rat thymocytes	Dallman et al, 1984
ST-1+	all ram T-cells	Miyasaka et al, 1985a
T-80+	ram T-helper/inducer lymphocytes	Miyasaka et al, 1983
ST-8+	ram cytotoxic lymphocytes	Miyasaka et al, 1985a
E-53+	ram B cells	Miyasaka et al, 1985a
SB-1+	ram class II MHC antigen	Miyasaka et al, 1985b

*: Kindly provided by Dr. A.F. Williams, Sir William Dunn School of Pathology, University of Oxford, U.K.
+: Kindly provided by Dr. M. Miyasaka, School of Medicine, Hamamatsu University, Japan.

serum.

4. Sections were washed 3 times with PBS.
5. Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:50 in PBS containing 1% bovine serum albumin, was incubated with sections for 30 minutes at room temperature.
6. Sections were again washed 3 times with PBS.
7. Sections were incubated in 3-3-Diaminobenzidine solution (see Farr and Nakane, 1981) for 5 minutes.
8. After another wash with PBS, slides were rinsed in distilled water.
9. Copper sulphate solution was applied to sections and incubated for 5 minutes.
10. Sections were again washed with PBS and rinsed in distilled water.
11. Mayers haemalum was used to counterstain sections which were then dehydrated in alcohol and mounted.

10.2.4. Semithin sections

Isolated testes of adult rats and rams were perfused through the testicular artery, initially with saline (rats 30 seconds, rams 2 minutes), and then with 5% gluteraldehyde + 3% p-formaldehyde in 0.2 M Cacodylate buffer, pH 7.2 (rats 15 minutes, rams 60 minutes). Pieces of testis of approximately 1 mm^3 were cut and immersed in the same fixative overnight. These were then washed in buffer, postfixed in 2 % osmium tetra-oxide, dehydrated in alcohol and embedded in Epon.

Semithin sections were cut at 1 um, stained with Toluidine Blue, and examined by light microscopy.

10.2.5. Paraffin sections

Pieces of rat and ram testis were fixed in 4% p-formaldehyde (see 10.2.3.1.) or Bouins fixative (see 2.14.1.). Tissue was then then embedded in paraffin as dscribed in 2.14.2., sectioned at 7 um, dehydrated, cleared and stained with toluidine blue to reveal metachromatic mast cells.

10.3. RESULTS

Histological sections after cytochemical staining are shown in Figure 10.1.a. Toluidine blue-stained semithin sections revealed cells with the morphological characteristics of macrophages in rat testes, as seen in Figure 10.1.a.1. These cells were relatively large and round in shape, and contained round nuclei bearing small indentations. The cytoplasm contained numerous densely stained granules and vacuoles. The cells were often associated with Leydig cells. No such cells were found in the toluidine blue-stained semithin sections of ram testis. Occasionally, elongated cells with irregular nuclei, were seen in the rat testis (Figure 10.1.a.2.). Similar cells were seen in the ram testis, with light coloured nuclei and a thin cytoplasm containing numerous granules.

In sections treated to reveal acid phosphatase activity, numerous large, round cells stained positive in the rat testis (Figure 10.1.a.3.). These cells were similar to those observed in the semithin

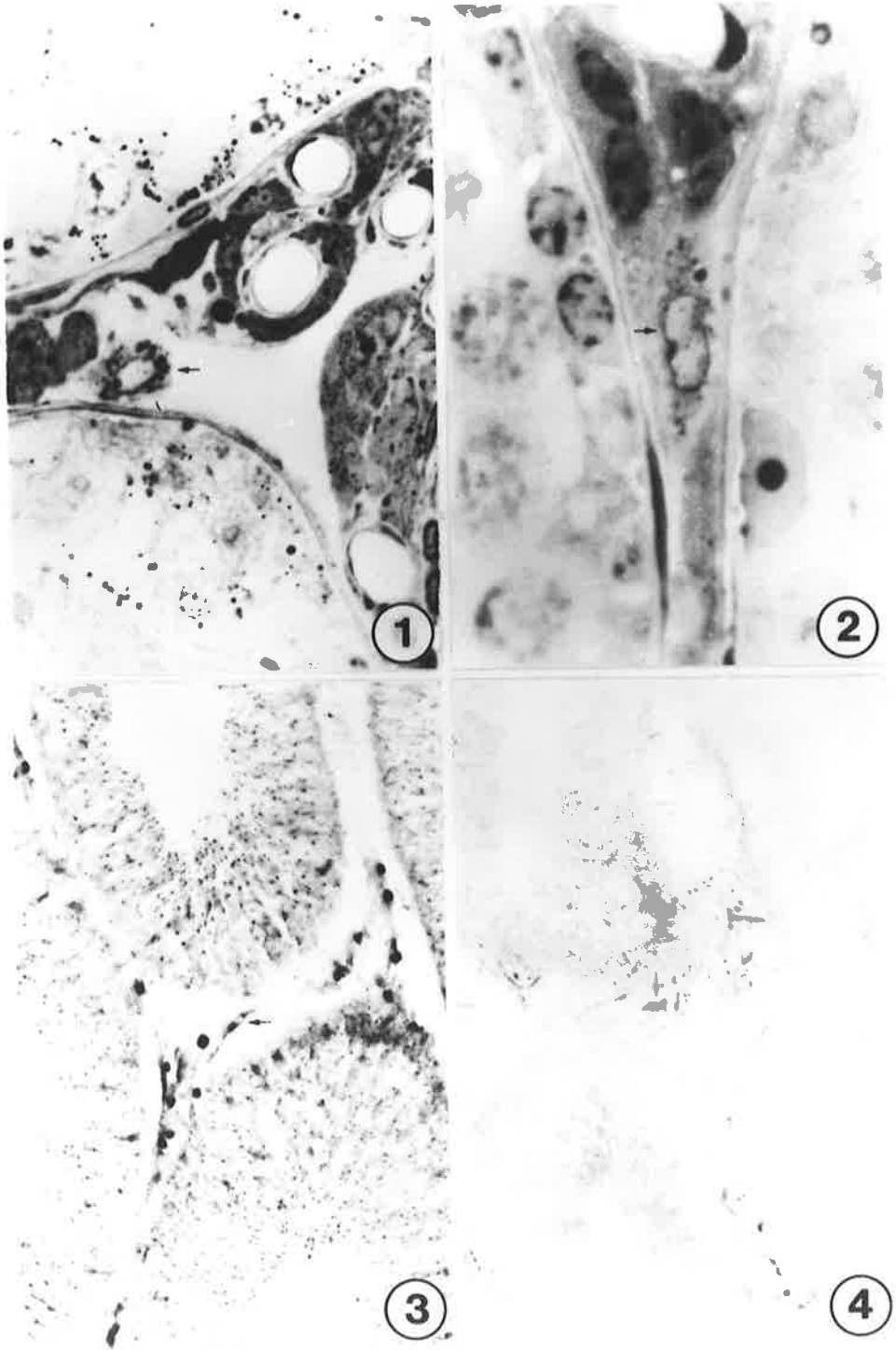
Figure 10.1.a Histological sections from the testes of rats and rams after cytochemical staining for immunocompetant cells.

(1) Toluidine blue staining in a semithin plastic section of a rat testis. Note the round macrophage (arrow) in the border of the lymph sinusoid and the interstitial cell cluster, and its close association with the adjacent Leydig cell. X 1300.

(2) Toluidine blue staining in a semithin plastic section of a rat testis. Note the elongated granule-filled macrophage (arrow). X 1500.

(3) Acid phosphatase activity in a para-formaldehyde-fixed frozen section of rat testis. Note the elongated positive cell (arrow). X 150.

(4) Acid phosphatase activity in a para-formaldehyde-fixed frozen section of ram testis. Only elongated positive cells are present in the ram testis (arrow). X 150.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sections and described above. As in the case of the semithin sections in the ram, no such cell were found in the sections of ram testis stained for acid phosphatase activity. Indeed, only a few phosphatase positive cells were seen in the ram testis, and these were elongated in shape and contained small granules (Figure 10.1.a.4.). Similar cells were also seen in the rat testis (Figure 10.1.a.3.)

In the rat, the round acid-phosphatase cells were scattered throughout the interstitium of the testis. The elongated cells seen in both ram and rat testes, appeared only in the interstitial tissue in both species, although occasional stained cells were found in close association with the lamina propria of the seminiferous tubules. Stained cells were never observed within the tubules in either species.

Macrophages of the rat testis comprised some 25 % of all interstitial cells, and formed the majority of phosphatase-positive cells. In both rat and ram, the elongated cells comprised some 1-2 % of the total interstitial cell population.

Figure 10.1.b. shows histological sections after indirect immunoperoxidase staining. T-lymphocytes (ram : ST-1+. rat : MRC Ox 19+) were only occasionally observed in testes of both the rat and the ram (Figures 10.1.b.5., 10.1.b.6.). They were usually found in close association with blood vessels, and were never seen in the tubules. Very few T-helper/inducer (T80+) or cytotoxic (ST-8+)lymphocytes were seen in the ram testis. B-lymphocytes (E-53+) were not found in the ram testis. No study was made of these cells in rat testis.

Major histocompatability antigen in rat testis (MRC Ox 17) was found on endothelial cells and macrophages (Figure 10.1.b.7.). The MHC

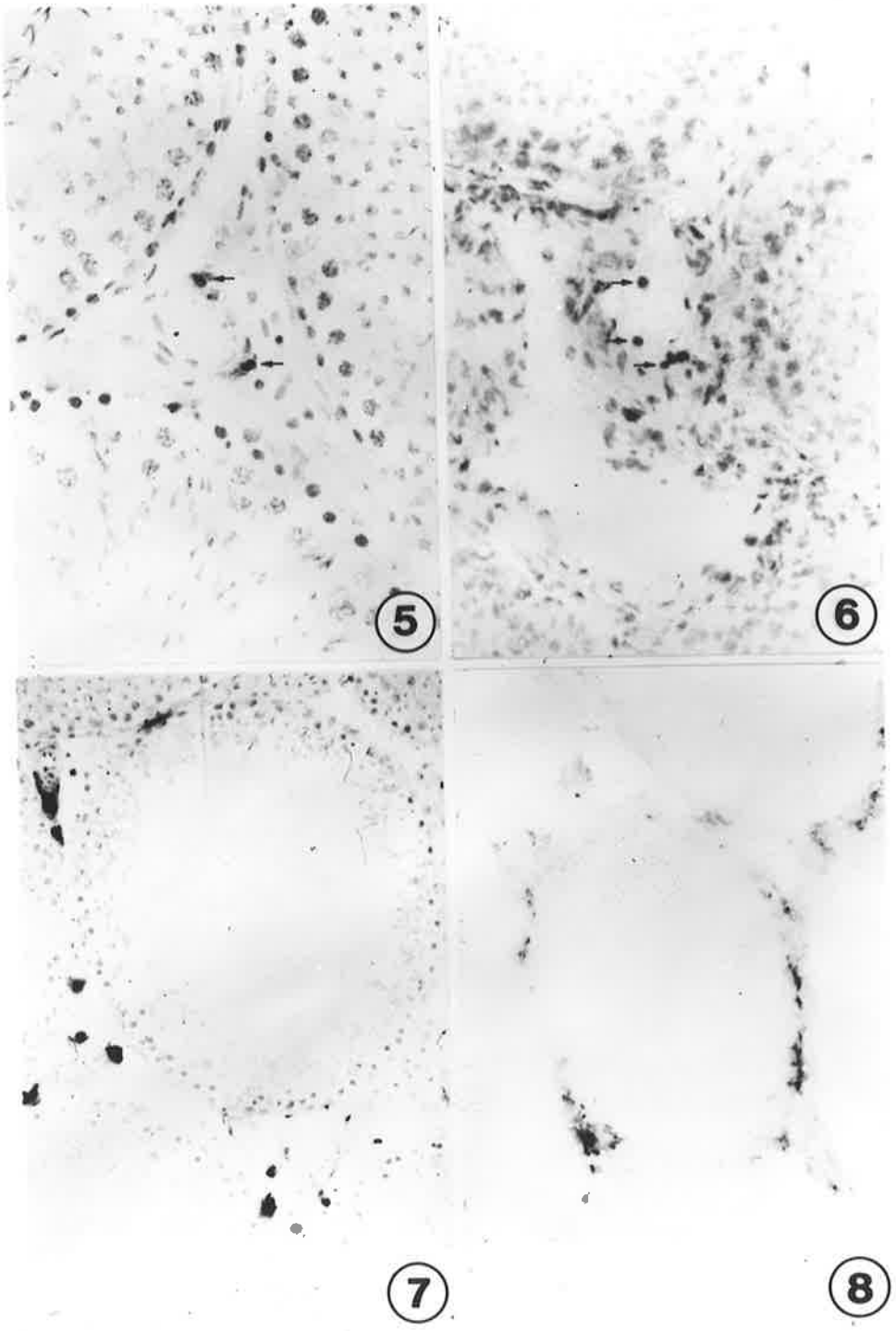
Figure 10.1.b Histological sections from the testes of rats and rams after indirect immunoperoxidase staining for immunocompetant cells.

(5) ST-1 positive T-lymphocytes in an acetone-fixed frozen section of rat testis, counterstained with Mayer's haemalum. X 500.

(6) ST-1 positive T-lymphocytes in an acetone-fixed frozen section of ram testis, counterstained with Mayer's haemalum. X 200.

(7) MRC Ox-17 positive Class II MHC antigen-positive cells in an acetone-fixed frozen section of rat testis, counterstained with Mayer's haemalum. X 150.

(8) SB-1 positive cells in an acetone-fixed frozen section of ram testis. X 150.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antigen in the ram testis (SB-1) was localised on the endothelial cells and occasional interstitial cells (Figure 10.1.b.8.). No antigen was found in the seminiferous epithelium of the rat, nor in the germinal epithelium of the ram.

No mast cells were identified on the basis of metachromasia, in toluidine blue-stained paraffin sections of rat or ram testis. Considerable numbers of mast cells have been demonstrated in the rat testicular capsule in the vicinity of the testicular artery by other investigators (see Sowerbutts et al, 1986) and other more specific methods of identification may be required.

Table 10.2. summarises the presence of various immune cells found in the course of the present study.

10.4. DISCUSSION

The present findings demonstrate differences in the magnitude and composition of the testicular macrophage population between the ram and the rat. Previous reports have identified macrophages in the rat testis by various means (Christensen, 1975; Bergh, 1985; Niemi et al, 1986), and found that they comprised some 25% of all interstitial cells. The figure obtained in the present study using cytochemical demonstration of acid phophatase activity, has confirmed these earlier reports. However, in contrast to these earlier reports, two morphologically different types of cells which exhibit acid phosphatase activity have been found in the rat testis . In addition to the large, round, phosphatase positive macrophages, a small proportion (1-2 %) of the interstitial cells were small and elongated, but exhibited acid phosphatase activity. Similar cells were also found in the ram testis, although no large round phosphatase positive macrophages were seen in ram testis. These small, elongated cells

Table 10.2. The immune cells demonstrated in the rat and the ram testis.

Cell Type	Rat Testis	Ram Testis
Macrophage		
large and round	+	-
small and elongated	+	+
Lymphocytes		
T cells	+	+
B cells	ND	-
Mast cells	-	-

ND = not determined

resemble the tissue histiocytes described by Stieve (1930) and may represent the stem cells of testicular macrophages and Leydig cells (Clegg and MacMillan, 1965a, b). While Paff et al (1947) reported mast cells to also exhibit phosphatase activity, no cells containing metachromatic granules were observed in the sections of the present study.

The observation that macrophages present in the rat testis are not found in the ram testis demonstrates a significant difference in the composition of immunocompetent cells in the testes of these two species. It is of great interest to speculate on the role of testicular macrophages in providing the immunologically privileged environment of the rat testis. Oehler et al (1977) have reported the rat spleen to contain a sub-population of macrophages which suppressed lymphocyte proliferation in mixed lymphocyte cultures. Similarly, Fernbach et al (1976) reported peritoneal-exudate macrophages to inhibit proliferation in mixed lymphocyte cultures in mice. Macrophage production of prostaglandin E_2 has also been shown to inhibit lymphoproliferation (Bray, 1980). Nelson (1976) has reviewed non-specific immunoregulation by macrophages and their products, and cites a number of cases where the inhibitory role of macrophages has been demonstrated. Hersey and MacLennan (1973) reported protection of certain tumour cells from lymphocyte toxicity by macrophages. Where target cells are killed solely or primarily by cytolytic effector T cells (as in the case of many tissue allografts) the presence of excess macrophages and/or activated macrophages can inhibit cytolysis (Kiessling et al, 1974; Vasudevan et al, 1974).

In 1982, Born and Wekerle reported that Leydig cells non-specifically suppressed mitogen- or allogeneic cell-induced

lymphoproliferation. They suggested that the Leydig cells, which have also been shown to adhere non-specifically to lymphocytes and leukaemic cells (Rivenson et al, 1974; Born and Wekerle, 1982), would provide an immunologically weak zone in the testicular interstitium - a region in which leukaemic cells often first reappear after chemotherapy (Pinkel, 1971). However, these authors characterised their Leydig cell preparations with criteria partly attributable to macrophages (Molenaar et al, 1984), and found 10-20 % non-Leydig cells. Testicular macrophages are often found in close association with Leydig cells and Miller et al (1983) reported portions of Leydig cell cytoplasm to be endocytosed by macrophages. The immunosuppressive activity of spleen derived macrophages in normal (Oehler et al, 1977) and leukaemic (Glaser et al, 1975) rats tempts one to suggest that the immunosuppressive activity reported for the above Leydig cell preaparations may have been caused by contaminating macrophages.

The interstitial macrophages of the rat testis have been shown to express class-II MHC antigen. The presence of SB-1 antigen in the ram is well correlated with the presence of MHC-II antigen (Dr Miyasaka, personal communication to Dr P. Pollanen) and cells bearing the SB-1 antigen are occassionally seen in the ram testis. The data obtained in the present study, suggests that the elongated cells expressing the acid phosphatase activity in ram testis are possibly macrophages, and might be capable of antigen expression to T-inducer lymphocytes. It is unlikely however, that they would act in the manner proposed for the large, round macrophages of the rat. The absence of MHC antigen in the germinal epithelium of the two species would aid in protecting the immunologically foreign germ cells from immune surveillance, since

initiation of an immune reaction would be impaired. Pollanen and Niemi (1987) have recently reported that the human germinal epithelium is devoid of MHC-II antigen.

T-lymphocytes have been found in the testis of both the ram and the rat, although they are few in number. Since these are the lymphocytes of consequence in allograft rejection, it is unlikely that difference in immune status of the testis in the ram and rat is due to differences in lymphocyte populations. Indeed, Head et al (1983c) have reported long-established intratesticular allografts in rats to be promptly rejected following specific, acute immunisation of the host, which would support this view.

The macrophage appears to be the one cell which shows major differences in the interstitial tissue of the rat and ram, and evidence has been presented which suggests that it may well be involved in localised immunosuppression. The physiological and immunological importance of Leydig cell-macrophage interaction remains to be determined, and further studies in this area are certainly warranted.

CHAPTER 11 : CONCLUSIONS

The studies reported in this thesis were initiated to re-examine the immunologically privileged status of the testis. Their pursuit has involved not only a study of the immunology of the endocrine testis, but a reassessment of aspects of the physiology of this region, particularly with regard to androgen secretion.

The testis of the rat has been confirmed as an immunologically privileged site, and thyroid and pituitary allografts have been found to survive for the three weeks of most studies, and longer (> 6 months in one instance). It does appear that vascularisation of tissue grafts in the testis is not as rapid as seen in other regions of the body, and this may be of importance in the immune privilege afforded this site. It may also explain the apparent lack of hormone secretion by tissue grafts examined 3 weeks after transplantation, when other indicators of graft function showed tissue viability.

The disruption of spermatogenesis by a number of experimental treatments did not alter graft survival. That thyroid allografts survived in abdominally-placed testes suggests that the lower temperature of the testis is not an important component of its immune privileged environment. During the present study, Head and Billingham (1985) also reported the survival of parathyroid allografts in abdominally-placed testes. These authors provided further evidence that temperature is not critical, when they demonstrated prompt rejection of parathyroid allografts placed in subcutaneous pockets in the ear - a site that is also of lower ambient temperature. While efferent duct ligation created significant damage to the seminiferous tubular epithelium, allograft survival was not affected. Sertoli cells are not morphologically altered with such treatment, although there is

evidence that the secretory functions of these cells are affected (see chapter 3). While the involvement of tubular elements could not be totally discounted, the results did suggest that factors responsible for immunosuppression were probably located outside the blood-testis barrier, within the interstitial tissue. Evidence that cellular associations/interactions in the interstitial region were probably important came from the findings that hCG-induced increases in vascular permeability and lymph flow had no effect on allograft survival.

A number of authors have cited the Leydig cells as possibly being responsible for immune regulation in the testis. Their role in steroid secretion and their apparent ability to adhere to lymphocytes are two attributes that would be useful in this regard. In the studies of this thesis, EDS was used specifically to destroy the Leydig cells. This led to rejection of thyroid allografts in the rat testis, confirming the involvement of these cells in maintaining an immune protection in this region. However the effects of Leydig cell destruction on the functions of other cell types in the testis were not assessed, and macrophages in particular have been shown to be closely associated with Leydig cells in the testis, and might well be altered by such treatments. It is disappointing that the use of intratesticular injections of silica specifically to destroy the macrophages was so unsuccessful. This technique appears to be of limited application in the testis since its effects are very localised, and other methods of macrophage destruction are needed.

A study of graft survival in the testis of the ram indicated that the immune status of the rat testis is not necessarily a general

mammalian characteristic. While thyroid autografts survived in the ram testis, and concentrated iodine 3 weeks after transplantation, thyroid and pituitary allografts were rejected within this time. These results led to an examination of the interstitial region of the testis in rats and rams to determine what differences existed, if any, that might explain the superior immune status of the ram testis.

The investigations of interstitial fluid required initial studies on the physiology of the endocrine testis. Available techniques for sampling interstitial fluid were found to be unsatisfactory, and somewhat unphysiological, and the Push-pull cannula used by neurophysiologists was adapted to investigate the interstitial fluid of the testis in rats, with a small trial involving rams and boars. A number of experiments were performed with the cannula, which resulted in the development of a new hypothesis on testosterone secretion in the testis. The studies suggested that Leydig cells secrete testosterone primarily into blood, and that interstitial fluid levels arise more as a consequence of blood levels. The first part of this proposal is in itself, not entirely new. In the early studies by Fawcett et al (1973), these authors stated that in the animals with interstitial organisation like that of the rat, the movement of testosterone from the Leydig cells could be envisaged as a release of androgens into blood capillaries and into the protein-rich extracellular fluids that move from the blood vascular system into the lymphatic sinusoids which surround the seminiferous tubules. They suggested that the lymphatic route of testosterone transport is the primary source of androgens for the tubules in these animals. In animals with interstitial arrangements like the ram and man, blood

capillaries are in much closer contact with the tubules and may supply testosterone directly to the tubules. In these species, lymphatics would be more involved in the return of extracellular fluids to the general circulation than in distribution of testosterone. The large mass of Leydig cells in the boar was presumed to be able to supply androgen directly to the tubules without the need for an indirect transport system such as the lymphatics or blood vascular system (see also Connell and Connell, 1977). Thus the potential importance of blood transport was recognised in the early anatomical studies. However it appears that the physiological importance of vascular transport of testosterone was lost in the reports of high testosterone levels in drip collected interstitial fluid (Hagenas et al, 1978; Sharpe et al, 1983; Turner et al, 1984). These reports appear to have been sufficient cause to neglect the potential problem of accounting for high interstitial levels of testosterone which can not be measured in testicular lymph or venous blood. The studies in the rat have also led to the hypothesis that significant levels of testosterone are required for spermatogenesis, and that these levels are provided for in interstitial fluid (Stevens and Steinberger, 1983). However, others report evidence to the contrary, suggesting that interstitial levels of 10-15% of "normal" can quantitatively maintain spermatogenesis (Cunningham and Huckins, 1979; Rea et al, 1986). Comhaire and Vermeulen (1976) make reference to studies reporting testosterone concentrations in interstitial tissue which exceeded the levels they found in "cell-free" interstitial fluid collected by micropuncture. They concluded that Leydig cells must contain considerable pools of testosterone which would contribute to levels measured in tissue. It is more than likely that these pools also contribute to the high

levels of testosterone measured in drip collected fluid. This study has hopefully provided sufficient incentive to ensure a re-evaluation of the contribution made by the vascular network in the testis for the distribution of testosterone.

The second part of the hypothesis developed in this thesis proposes that interstitial fluid levels of testosterone arise more as a consequence of blood levels. The studies with the push-pull cannula demonstrated that interstitial fluid levels of testosterone are no greater than testicular venous blood levels, and may in fact be somewhat less; and are also more comparable to those levels found in testicular lymph. Results from studies using such treatments as heat, hCG injections, and testosterone implants to alter testicular function, have also suggested that blood flow and capillary permeability may be important factors in determining interstitial fluid levels of testosterone. These factors are also regulated by cells in the seminiferous tubules and change in relation to various stages of spermatogenesis (Setchell and Galil, 1983; Bergh and Damber, 1984; Bergh 1985). The present studies, with support from the literature, suggest that testosterone requirements for spermatogenesis are not met by excessively high levels of the hormone in interstitial fluid as previously assumed (see above). The seminiferous tubules undoubtably require greater levels of testosterone than are achieved elsewhere in the body, and levels of testosterone in testicular interstitial fluid are certainly greater than the extracellular fluid levels anywhere else in the body. However, it seems that these levels are much lower than previously thought. Requirements of testosterone for continued spermatogenesis seem to be controlled by intricate regulation of blood flow and capillary permeability, and close

paracrine communication between Leydig cells and the seminiferous tubules.

The level of testosterone in the interstitial fluid of rams and boars was found to be similar to that in the rat, and no evidence could be found in the literature to suggest that the steroid profile of rams and rats, or the levels of steroids involved would differ sufficiently to account for the different immune status of the testis in these two species. Further studies are obviously required before the lack of steroid involvement can be generally concluded. However, from the available information, the present studies do suggest that the importance of the Leydig cell for the immune privilge of the rat testis is not necessarily because of its steroid secretion. The interest in immunosuppression by steroid hormones has largely arisen as a hypothesis to account for survival of the fetus in the womb - it is after all an allograft. However, the subject is somewhat controversial. According to the in vitro studies of Pavia et al (1979), progesterone and oestradiol were effective inhibitors of cell-mediated lympholysis in mice at concentrations of 5 -10 ug/ml. Cortisol was also effective at these concentrations, but testosterone was not at all immunosuppressive. Part of the controversey of the role of sex steroids in immunosuppression during pregnancy revolves around physiological versus pharmacological concentrations. For example, the levels of progesterone required for immunosuppression in mice cells in the study by Pavia et al (1979) are unphysiologically high. Even in the pregnant mouse when progesterone levels should be at their greatest, circulating levels are only 6 - 70 ng/ml (Parkening et al, 1978). It is certain that reproductive steroids do have cytotoxic effects on lymphocytes at dose ranges of 2 - 50 ug/ml (Kitzmiller and

Rocklin, 1980). However, it is also apparent that steroids alone cannot account for the immunosuppressive effect of pregnancy serum (Schiff et al, 1975). If this is a problem in pregnancy-related immunosuppression, then it is an even greater problem for immunosuppression in the testis. The required levels of these hormones are unlikely to be found in the interstitial tissue. Certainly, the measurements made with the push-pull cannula need to be extended to cover other steroids. However, if the testosterone results are in any way related to the levels of other steroids, the push-pull data would suggest that there are going to be even lower levels than previously anticipated. In summary, the data obtained from the present study, in conjunction with numerous literature reports, casts severe doubt on physiological role of steroids as modulators of host responsiveness to tissue allografts in the testis. Furthermore, steroid immunosuppression tends to prevent lymphocyte proliferation, and as discussed by Kitzmiller and Rocklin (1980), these effects in vivo would not inhibit the effector part of the immune response. Inhibition of this arm of the response is important for graft survival in the testis, since Head and Billingham (1983) have demonstrated rejection of viable intratesticular allografts following extra-testicular sensitisation of the host against the graft donor - an efferent response. Therefore, other mechanisms are required to explain the success of tissue allografts in the testis, and for that matter the success of the fetus in the womb of a presensitised host; and these mechanisms should inhibit an effector, not a proliferative, response.

The reports of macrophages in the interstitial region of the testis, and their close involvement with Leydig cells prompted an

investigation of the immunocompetant cells of the rat and ram testis. This investigation revealed that rams do not posses the large, round macrophages found in the rat testis. A number of literature reports have provided evidence for an immunosuppressive role of tissue-specific macrophages. As discussed in chapter 1, initiation of an effector response by the immune system is dependent on macrophages processing of antigen presented in the afferent immune response. Macrophages in the testis may process antigen locally without subsequent presentation to effector cells, and thereby significantly reduce the strength of the afferent response. Indeed, under such circumstances tolerance may be induced, rather than rejection. However, the effectiveness of extratesticular sensitisation in initiating graft rejection suggests that a state of tolerance may not necessarily exist. Cytotoxic T-cell mediated cytolysis of target tissues can also be prevented by the presence of large numbers of macrophages, as discussed in chapter 11. This cytotoxic T-cell response is also a component of the efferent response of the immune system - the response that needs to be controlled according to Kitzmiller and Rocklin (1980). It is the conclusion of this thesis, that the macrophages of the rat testis, probably in conjunction with the Leydig cells, are of key importance in providing an immunologically privileged environment in the rat testis. The mechanisms involved in this immunoregulation and the significance of Leydig cell involvement have yet to be determined. However the intimate association of Leydig cells with macrophages, may affect macrophage function and explain why allografts were rejected after Leydig cell destruction with EDS. That the ram does not appear to contain testicular macrophages of the type found predominantly in the

rat testis, and rejects tissue allografts prompts further investigations in other species. However, it may mean that the potential use of tissue allografts in agriculturally important species to stimulate growth and modify aspects of endocrine function is limited. The recent report of macrophages in the human testis which are similar to those in the rat testis (Pollanen and Niemi, 1987) means that the use of the human testis as an immunologically privileged site, remains an exciting prospect.

CHAPTER 12: BIBLIOGRAPHY

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