THE DISTRIBUTION AND METABOLISM OF CREATINE IN
THE RAT TESTIS: IN VIVO AND IN VITRO EFFECTS OF
TESTICULAR TOXICANTS

NIGEL PHILLIP MOORE B.Sc. (Hons)

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Doctor of Philosophy

Department of Toxicology,
The School of Pharmacy,
The University of London,
29-39 Brunswick Square,
LONDON WCIN 1AX.
Sherlock Holmes: "It is an old maxim of mine that when you have excluded the impossible, whatever remains, however improbable, must be the truth."

ABSTRACT

Early work in this laboratory has shown that after testicular damage is induced in the rat there is an increase in the excretion of creatine in the urine. The aim of this project was to examine the distribution and metabolism of creatine within the rat testis, and to assess urinary creatine excretion as an index of testicular damage.

Groups of rats were administered single doses of various cell-specific testicular toxicants: a germ cell toxicant (methoxyacetic acid, MAA), one of two Sertoli cell toxicants (di-n-pentyl phthalate, DPP; 1,3-dinitrobenzene, 1,3-DNB), or a Leydig cell toxicant (ethane-1,2-dimethane sulphonate, EDS). Urinary creatine and creatinine levels were monitored over the following 48 h, after which time the testes were removed, weighed and, after processing, sections were examined by light microscopy. All four treatments resulted in reductions in relative testis weight and produced morphological changes similar to those which have been widely reported in the literature. In addition, MAA, DPP and 1,3-DNB all caused large, transient elevations in urinary creatine excretion and the urinary creatine:creatinine ratio within 24 h. EDS had no such effect. It is inferred from these observations that creatine is associated with the seminiferous epithelium, and may represent a marker for damage to these cells in vivo.

The distribution of creatine within the seminiferous epithelium was examined by the use of Sertoli and germ cells isolated from the testes of 4 week old rats. Both cell populations were found to contain creatine and N-phosphorylcreatine (PCr) pools, and to express creatine phosphoryltransferase (CPT) activity.

Isolated seminiferous tubules synthesised creatine, and its biosynthetic precursor, guanidinoacetic acid (GAA), from L-arginine as determined by radio-metabolism studies using L-[guanidino-14C]arginine. A crude interstitial cell preparation synthesised neither creatine nor GAA. Cultured Sertoli cells incorporated radioactivity from both L-[guanidino-14C]arginine and [6-14C]glycine into creatine and GAA. Therefore, Sertoli cells have the
capacity to carry out both stages of creatine synthesis; transamidination between arginine and glycine, with subsequent methylation of GAA to creatine. Germ cells did not exhibit this activity.

Cultured Sertoli cells, maintained in a defined medium, secreted creatine into the overlying incubation medium, in a manner which was linear with time over at least 6 h, but which reached a plateau within 24 h. The secretion of creatine over 24 h was stimulated both by physiological and toxicological modulators of Sertoli cell function. Stimulation of creatine secretion by follicle-stimulating hormone (FSH) and \( N^8, O-2'-\)dibutyl adenosine 3',5'-cyclic monophosphate (dbcAMP) was enhanced by the inhibition of adenosine 3',5'-cyclic monophosphate phosphodiesterase (cAMP PDE), indicating that creatine secretion is under the influence of intracellular cAMP levels. The secretion of creatine by cultured Sertoli cells, incubated over 4 h in EBSS, was independent of exogenous L-glutamine. However, the dbcAMP-induced stimulation of creatine secretion was dependent upon the presence of L-glutamine in the incubation medium, suggesting that increases in creatine secretion may occur as a consequence of stimulated glutamine oxidation.

Isolated germ cells sequestered \([^{14}C]creatinine\) in a time-dependent manner, which was linear over at least 3 h, by a two component process. One component had a high affinity for creatine \( (K_m = 25 \mu M)\), was inhibited both at low temperature and by the absence of sodium ions from the medium, and probably represents an active uptake system. The other component, which had a low affinity for creatine and was independent of temperature and sodium, probably represents passive diffusion.

It is concluded that an intercellular pathway for creatine metabolism may exist within the seminiferous epithelium of the rat, by which creatine is synthesised and secreted by the Sertoli cells and is taken up by the germ cells from the interstitial milieu. Creatine is phosphorylated within both the Sertoli and germ cells, but its role is as yet unclear.
ACKNOWLEDGEMENTS

I would like to thank my supervisors, Drs. John Timbrell and Tim Gray, for their guidance and support over the period of this project, and Dr. Elizabeth Moss for introducing me to the world of toxicological research in both my B.Sc. and Ph.D. degree courses.

There are many others who have aided (and abetted!) my efforts and to whom I owe a sincere debt of gratitude. Dr. Dianne Creasy at BIBRA, LSR, Shell Research etc. for her histological insight and useful discussions, and Dave McCarthy at SoP for processing samples for histological examination and teaching me the rudiments of staining techniques (!). Angela Savage and Mel Cook at BIBRA for demonstrating the intricacies of cell culture and for useful discussions. Dave Walters at BIBRA for insight into the world of HPLC. Members of the Chemistry Department at SoP, in particular Wilf, for their kind assistance, use of equipment, putting up with me.... The computer wizards, Graham Florence at SoP and Spencer O'Dea at BPN CPD.

A particular vote of thanks goes to my fellow "Poxy Toxies": Simon, Colin, Steve, Jane, Tim, and Andy and Michael, my ace singing partners. Also to Fiona, Dyane and Alan, without whose help and encouragement it is doubtful that this thesis would have ever seen the light of day.

Finally, I would like to thank my parents for all their love and support over the years, and without whom this would not have been possible. It is to them that this thesis is dedicated.
# ABBREVIATIONS

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<tr>
<td>ABP</td>
<td>androgen binding protein</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CPT</td>
<td>creatine phosphoryltransferase (ATP: creatine N-phosphoryltransferase)</td>
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<tr>
<td>Cr:Crn</td>
<td>creatine:creatinine molar ratio</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>N⁶,O-2'-dibutyl adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNB</td>
<td>dinitrobenzene</td>
</tr>
<tr>
<td>DNT</td>
<td>dinitrotoluene</td>
</tr>
<tr>
<td>DPP</td>
<td>di-n-pentyl phthalate</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle's balanced salt solution</td>
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<tr>
<td>EDS</td>
<td>ethane-1,2-dimethane sulphonate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle's minimal essential medium</td>
</tr>
<tr>
<td>(p)FSH</td>
<td>(porcine) follicle-stimulating hormone</td>
</tr>
<tr>
<td>G protein</td>
<td>guanylate nucleotide binding protein</td>
</tr>
<tr>
<td>GAA</td>
<td>guanidinoacetic acid</td>
</tr>
<tr>
<td>GAT</td>
<td>glycine amidinotransferase</td>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
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<tr>
<td>GMT</td>
<td>guanidinoacetate methyltransferase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione (reduced)</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>-------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin stain</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>LDH(-X)</td>
<td>lactate dehydrogenase(-X (also -C₄))</td>
</tr>
<tr>
<td>LH</td>
<td>luteinising hormone</td>
</tr>
<tr>
<td>MAA</td>
<td>methoxyacetic acid</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-methoxyethanol (ethylene glycol monomethyl ether)</td>
</tr>
<tr>
<td>MEHP</td>
<td>mono(2-ethylhexyl) phthalate</td>
</tr>
<tr>
<td>MIX</td>
<td>3-isobutyl-1-methylxanthine</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>NAD(H+/−)</td>
<td>nicotinamide adenine dinucleotide (reduced / oxidised)</td>
</tr>
<tr>
<td>NADP(H+/−)</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced / oxidised)</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>PBSG</td>
<td>phosphate buffered saline with D-glucose</td>
</tr>
<tr>
<td>PES</td>
<td>phenazine ethosulphate</td>
</tr>
<tr>
<td>PCr</td>
<td>N-phosphorylcreatine</td>
</tr>
<tr>
<td>PIA</td>
<td>N⁶-(2-phenyl-iso-propyl) adenosine</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SCEC</td>
<td>Sertoli cell-enriched cultures</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SGCC</td>
<td>Sertoli-germ cell co-cultures</td>
</tr>
<tr>
<td>SDH</td>
<td>succinate dehydrogenase</td>
</tr>
<tr>
<td>T₃</td>
<td>triiodothyronine</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>tricarboxylic acid (also Krebs or citric acid) cycle</td>
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Tris-HCl  tris(hydroxymethyl) aminomethane hydrochloride
TSH  thyroid stimulating hormone
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CHAPTER 1

INTRODUCTION
1.1  THE TESTIS.

1.1.1  Testicular structure and function.

The testes are paired ovoid organs that serve the dual function of producing both the male gametes and steroid sex hormones. These two activities occur in morphologically distinct compartments, the seminiferous epithelium and the interstitium.

Around the time of birth, the mammalian testes descend from the abdomen into the scrotum, where they are maintained at a few degrees below body temperature. Failure to descend results in cryptorchidism, characterised by degeneration of spermatogenic cells and alterations in the functional parameters of the somatic cells of the testes. The effects of cryptorchidism appear to be solely due to the effect of heat, since they can be duplicated by warming the testes or by surgically transferring the testes to the abdomen.¹

The testis is supplied with blood from the internal spermatic artery via the testicular artery. The internal spermatic artery follows a tortuous and convoluted path in which it is immediately juxtaposed with the testicular vein in the pampiniform plexus.² This arrangement serves as a heat exchanger, cooling the afferent blood supply (by up to $5.2 \, ^\circ\text{C}$ in the ram).¹ ² The scrotum also aids temperature regulation in the testes by controlling their proximity to the body, and by allowing heat exchange with the atmosphere.¹

The seminiferous epithelium (§1.1.1a) is arranged into tubular structures, and is the site of germ cell development and maturation. Associated with the germ cells are the Sertoli cells, which physically and biochemically support and regulate the process of spermatogenesis (§1.2).

Surrounding the tubules are the myoid cells. These are smooth muscle cells whose contraction moves the non-motile spermatozoa through the tubules towards the rete testis, from which point they enter the epididymis. The myoid cells are also thought to be involved in the regulation of activity within the seminiferous epithelium, and constitute part of the blood-testis barrier (§1.2.1ai).

The interstitium comprises the Leydig cells (§1.1.1c), blood and lymph vessels,
nervous tissue, fibrous connective tissue, lymphocytes and macrophages. The principal function of the interstitium is to allow the exchange of nutrients and waste products with the blood, and the production of the male sex hormones.

1.1.1a The seminiferous epithelium.

The seminiferous epithelium is a close association of somatic (Sertoli) cells and germ cells at different stages of maturation. As the germ cells mature, they pass from the basal region of the epithelium towards the lumen, when eventually as testicular spermatozoa they are shed from the epithelium and pass down the lumen to leave the testis altogether.

The principal functions of the Sertoli cells are the maintenance and regulation of spermatogenesis, offering mechanical and nutritional support to the germ cells. In addition, tight intercellular junctions between neighbouring Sertoli cells form the blood-testis barrier, which maintains a strict control of the environment within the tubule. These functions are described more fully below (§1.2.1).

1.1.1b Spermatogenesis.

Spermatogenesis is a process that follows a strict series of temporal events that can be divided into three distinct phases: spermatocytogenesis, in which spermatogonia divide to give rise to spermatocytes and simultaneously maintain their population by renewal; meiosis, in which spermatocytes duplicate their DNA complement and then undergo the two successive divisions that result in the formation of haploid cells, the spermatids; and spermiogenesis, in which the spermatids undergo morphological maturation, resulting in spermatozoa which are shed into the lumen, and which then pass out of the testis altogether.1,4

Spermatogenesis is initiated at precise intervals by the division of stem cells, the type A spermatogonia. Through successive divisions, the first generation of spermatogonia (type A₁) gives rise to further generations (types A₂, A₃, A₄, intermediate, and B). Division of
the type B spermatogonia yields the preleptotene spermatocytes which are at the interphase stage of the first meiotic division. Type A, spermatogonia are replenished in order to maintain the stem cell population, but the precise mechanisms involved are as yet unclear.\textsuperscript{4,6}

The preleptotene spermatocytes duplicate their DNA content, and then enter the prophase of the first meiotic division as leptotene spermatocytes. Nuclear and cytological changes progressively take the cells through the zygotene and pachytene stages of the first prophase, until at the end of the diplotene stage the tetraploid nucleus goes through metaphase, anaphase, and telophase of the first maturation division to yield diploid secondary spermatocytes. These cells are short-lived, and without duplicating their DNA they enter the second maturation division, yielding the haploid round spermatids.\textsuperscript{4}

Spermatids undergo a series of complex cytological and nuclear alterations, during which the cells elongate and develop flagellae, and their nuclei condense.\textsuperscript{7} Finally the cytoplasm and non-essential organelles, which constitute the residual bodies, are shed and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{spermatogenic_cycle.png}
\caption{The spermatogenic cycle in the Sprague-Dawley rat. The framework of the diagram gives the cellular composition of the 14 stages of the cycle (I-XIV). The widths of the stages are proportional to their relative durations. A, In, B; type A, intermediate type, and type S spermatogonia; Pl, L, Z, P, Di, II: preleptotene, leptotene, zygote, pachytene, diplotene, and secondary spermatocytes; 1-19 stages of spermiogenesis. Modified from Clermont.\textsuperscript{4}}
\end{figure}
the spermatozoa are released into the lumen, a process that is termed spermiation.\textsuperscript{4}

Maturing germ cells are arranged in well defined cell associations or stages, fourteen of which have been described in the rat. These cell associations are comprised of one or two generations each of spermatogonia, spermatocytes, and spermatids (Figure 1.1), which succeed one another in time at any given point along the seminiferous tubules in a specific order. Thus stage I in the rat is followed by stage II and preceded by stage XIV. This phenomenon is known as the cycle of the seminiferous epithelium, and is of constant duration for any particular species (approximately 13 days in the rat).\textsuperscript{4} Different stages of spermatogenesis are present within the seminiferous epithelium at any particular time, and therefore spermatogenesis proceeds as waves down the length of the tubules.\textsuperscript{4,5}

\textbf{1.1.1c The Leydig cell.}

The Leydig cells lie within the interstitium and are the site of synthesis of the male sex hormone testosterone (17β-hydroxyandrost-4-en-3-one) within the testis. They are large polyhedral cells with an extensive smooth endoplasmic reticulum, which is characteristic of steroid-secreting cells.\textsuperscript{1,3}

Testosterone is secreted by the Leydig cells and, in the rat, it is carried to its target cells by a specific protein carrier, androgen-binding protein (ABP), originating from the neighbouring Sertoli cells.

\textbf{1.1.2 Regulation of testicular function.}

Testicular function is regulated by the hypothalamus via hormonal interactions with both the Leydig and Sertoli cells (Figure 1.2). Gonadotropin-releasing hormone (GnRH), released from the hypothalamus, stimulates the release of two glycoprotein hormones, luteinising hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary.\textsuperscript{1,6}

Cellular function within the testis is also under complex paracrine and autocrine regulation.
Figure 1.2. The regulation of testicular function. Gonadotropins, released from the anterior pituitary under the influence of gonadotropin-releasing hormone (GnRH), interact with the Sertoli and Leydig cells to regulate their activity. Interactions between different populations of testicular cells also take place. See text for details. This diagram represents a summary of some of the more well established pathways. Other less well defined pathways have been omitted for clarity.
1.1.2a Regulation of the seminiferous epithelium.

The seminiferous epithelium is under complex hormonal control, mediated by both FSH and androgens. This is amply demonstrated by hypophysectomy which results in complete inhibition of spermatogenesis, and atrophy of the testes and secondary sexual organs. These effects can be prevented or reversed by administering pituitary extracts. FSH is required for the initiation of spermatogenesis at puberty or after hypophysectomy. However, once spermatogenesis has been initiated it can be maintained by the administration of LH or testosterone alone, although under these conditions spermatogenesis is not quantitatively normal. The somatic cells of the seminiferous epithelium, the Sertoli cells, seem to be the primary site of action for both FSH and testosterone, although the presence of androgen receptors within the germ cells suggests that they may be under direct hormonal control also.

FSH binds to specific receptors upon the Sertoli cell surface that are linked to adenylate cyclase (ATP pyrophosphate-lyase). Stimulation of Sertoli cells by FSH, results in an increase in intracellular levels of adenosine 3',5'-cyclic monophosphate (cAMP), which is the secondary messenger in the hormone signalling system (§ 1.2.2ai).

The proliferation of the Sertoli cells during the early development of the testis may be under the influence of FSH, since it stimulates DNA synthesis and mitotic activity in immature rat Sertoli cells. However, this effect is age-dependent, declining rapidly after 20 days of age in the rat, with no effect at 40 days. In addition to stimulating the proliferation of Sertoli cells, FSH also stimulates mitotic activity in the primordial germ stem cells and spermatogonia of one week old rats, although this effect may be mediated by the Sertoli cells. Again there is no effect after three weeks, further implying that FSH is required for the initiation of germ cell proliferation, but not for its maintenance.

Age-dependency is a feature of all intracellular events stimulated by FSH in isolated Sertoli cells, with activity peaking at around three weeks of age. This is the time at which tight junctions between Sertoli cells first appear in vivo, and coincides with the onset of
meiosis, and therefore complete spermatogenesis, in the rat. The reduction in the response of the seminiferous epithelium to FSH seems not to be due to a loss of FSH receptors but to an increase in cAMP catabolism, since the binding capacity of the tubules is not lost, the responsiveness to N\(^6\),O-2'-dibutyl cAMP (dbcAMP), a lipid-soluble cAMP derivative, is also reduced, and FSH responsiveness can be returned by inhibiting cAMP catabolism.\(^{17,18}\)

FSH induces many functional alterations in the Sertoli cells (§1.2.2ai), but two are particularly important in relation to pituitary-testicular regulatory activity. Firstly, FSH increases the secretion of ABP, which carries testosterone into the seminiferous tubules and, via the bloodstream, to its other target cells, including those secreting LH in the pituitary (§1.1.2b). Secondly, FSH stimulates the secretion of a protein hormone, inhibin, which inhibits further release of FSH from the anterior pituitary. Cultured rat Sertoli cells synthesise and secrete inhibin, and this secretion is enhanced by the presence of FSH in the culture medium.\(^{21-23}\) Further, the suppression of serum and testicular inhibin concentrations after hypophysectomy suggests that FSH stimulates inhibin secretion in vivo also.\(^{20}\) In vivo inhibin is carried via the bloodstream to the pituitary, where it inhibits further release of FSH,\(^1,24,25\) and experiments with cells cultured from the anterior pituitary show that inhibin inhibits the secretion of FSH by these cells,\(^{24}\) possibly by inhibiting FSH synthesis.\(^{26}\) Thus FSH release from the anterior pituitary, like that of LH, is regulated by a long loop feedback mechanism.

Testosterone, secreted by the Leydig cells, enters the seminiferous epithelium and interacts with two proteins. The first functions as the Sertoli cell cytosolic androgen receptor and mediates testosterone activity within the epithelium.\(^6,27\) The second (ABP) is secreted by the Sertoli cells and transports testosterone and its primary metabolite, 5α-dihydrotestosterone, within the seminiferous epithelium and lumen of the tubule, and in the rat it transports testosterone in the bloodstream.\(^6\)

Androgen receptors have been discovered in both the cytosolic and nuclear compartments of the rat Sertoli cell,\(^9,11\) and in the nuclear compartment of rat germ cells.\(^{11}\) These observations suggest that both cell types associated with the seminiferous epithelium
are targets for androgens, although the role(s) of these hormones and the mechanisms involved are as yet poorly understood.

The control of Sertoli cell function is further discussed in §1.2.2.

1.1.2b Regulation of Leydig cell function.

The principal function of the Leydig cells is the production of androgens. This is under the influence of LH, which stimulates androgen synthesis by enhancing the conversion of cholesterol to pregnenolone.\(^1,6,8\) The testosterone that is secreted by the Leydig cells passes from the interstitium into both the seminiferous epithelium and the bloodstream, carried by ABP which is synthesised by the Sertoli cells under the influence of both testosterone and FSH.\(^9\) Testosterone inhibits the further release of LH by a feedback mechanism in the adenohypophysis.\(^1,6\)

In addition, the Sertoli cells have been implicated in the regulation of Leydig cell function. Cultured immature porcine Sertoli cells secrete factor(s) that inhibit the gonadotropin-induced stimulation of testosterone secretion by cultured immature porcine Leydig cells, although basal testosterone secretion is unaffected.\(^28\) These effects are mimicked by transforming growth factor, and a similar peptide has been shown to be secreted by cultured porcine Sertoli cells.\(^29-31\) Therefore, testosterone secretion by Leydig cells may be under control by the Sertoli cells, although the full physiological mechanisms and significance of this are as yet unclear.

1.1.3 The testis as a target for toxic action.

Male reproductive function is a highly complex process, requiring the co-ordinated interaction of a wide variety of cell types and hormones. It can therefore be disrupted at many levels,\(^32-35\) and a brief summary of toxicants that affect male reproduction is given in Table 1.1. However, for ease of discussion, only a selection of those toxicants that are considered to affect the testis directly will be discussed in this section.
Table 1.1. Agents reported to disrupt male reproductive capacity. This table gives a brief summary of the wide range of compounds that affect male reproductive function. It is not intended to be an exhaustive list. Adapted from Dixon and Chambers et al.\textsuperscript{32, 33}

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<tr>
<th>Category</th>
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<td>Anti-gonadotropin antibodies</td>
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<td>Steroids</td>
<td>Anti-androgens</td>
<td>cyproterone acetate</td>
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<td></td>
<td>Oestrogens</td>
<td>spironolactone</td>
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<td></td>
<td>Progestogens</td>
<td></td>
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<tr>
<td></td>
<td>Inhibitors of steroid synthesis</td>
<td></td>
</tr>
<tr>
<td>Alkylating agents</td>
<td>Nitrogen mustards</td>
<td>cyclophosphamide, chlorambucil</td>
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<tr>
<td></td>
<td>Nitrosoureas</td>
<td>1,3-bis(2-chloroethyl)-1-nitrosourea</td>
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<tr>
<td></td>
<td>Esters of methane</td>
<td>EDS, busulphan</td>
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<td></td>
<td>sulphonic acid</td>
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<td></td>
<td>Hydrazine derivatives</td>
<td>procarbazine</td>
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<td>Ethylenimines</td>
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<td>Metabolic inhibitors</td>
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<td>Nucleic acid analogues</td>
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<td>Glucose antagonists</td>
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<td>Drug abuse</td>
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<td>Physical factors</td>
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<td>Radiation</td>
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<td></td>
<td>Stable isotopes</td>
<td>deuterium oxide</td>
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1.1.3a Ethylene glycol ethers.

The monoalkyl ethers of ethylene glycol are an important class of solvents that are miscible with both water and many organic solvents. In rats and mice they induce testicular atrophy, foetal malformation, bone marrow suppression, and thymic atrophy.\(^{36,37}\)

Testicular toxicity as a result of exposure to glycol ethers has been recognised for many years, Wiley *et al.* first described degeneration and desquamation of the seminiferous epithelium in rabbits treated with 2-methoxyethanol (2-ME, ethylene glycol monomethyl ether).\(^{38}\) Later studies in rats and mice demonstrated that 2-ME is the most potent testicular toxicant of the class, with 2-ethoxyethanol being substantially less toxic, and ethylene glycol and higher homologues having no effect.\(^{39-42}\) The testicular lesion induced by 2-ME in rats is characterised by a dose-related, specific degeneration of the early (stages I-II) and late (stages XII-XIII) pachytene and dividing (stage XIV) spermatocytes.\(^{39,43,44}\)

The glycol ethers are oxidised *in vivo* by alcohol dehydrogenase and aldehyde dehydrogenase to the corresponding alkoxyacetic acids,\(^{39,45-49}\) which are in turn conjugated with glycine,\(^{45-48}\) presumably via an intermediary coenzyme A derivative, alkoxyacetyl coenzyme A. Thus 2-ME is oxidised to methoxyacetic acid (MAA), which is conjugated with glycine to produce N-methoxyacetylglucose.\(^{39,45,48,49}\) MAA and N-methoxyacetylglucose constitute the major urinary metabolites of 2-ME in the rat.\(^{45}\)

The role of metabolism in the development of 2-ME-induced testicular toxicity has been well established, both *in vivo* and *in vitro*. The administration of pyrazole, an inhibitor of alcohol dehydrogenase activity, to rats prior to 2-ME treatment prevents not only the oxidation of 2-ME to MAA, but also the induction of testicular damage.\(^{39,45}\) Further, MAA induces a similar testicular lesion to that of its parent glycol ether when administered to rats.\(^{39,40,50,51}\) In addition, the exposure of mixed testicular cell cultures to MAA results in the specific degeneration and exfoliation of pachytene spermatocytes,\(^{39,51-53}\) whereas 2-ME, which is not oxidised by these cultures, and N-methoxyacetylglucose are without effect.\(^{52,53}\)

Further evidence for the role of metabolism in glycol ether-induced testicular toxicity
is derived from the comparison of metabolism and toxicity for 2-ME and 2-methoxy-1-methylethanol \((\text{iso-propylene glycol monomethyl ether})\), which differs from 2-ME by a single methyl group. 2-methoxy-1-methylethanol is a secondary alcohol, and as such is a poor substrate for alcohol dehydrogenase. When given to rats it is not metabolised by the same route as 2-ME, is not oxidised to an alkoxy acid, and does not induce testicular damage.\(^{48,49}\)

The mechanism by which MAA exerts its toxic effects upon the pachytene spermatocyte population has not as yet been fully resolved. Initial studies suggested that the Sertoli cell was a locus for toxic action by MAA, stimulating lactate oxidation and reducing lactate secretion,\(^ {54,56}\) presumably resulting in germ cell death by withdrawal of this essential energy-yielding substrate (§1.2.1bi). However, the supplementation of testicular cell co-cultures with lactate does not prevent the MAA-induced degeneration of pachytene spermatocytes,\(^ {52}\) and MAA also reduces the viability of pachytene spermatocytes cultured in a defined medium and in the absence of Sertoli cells.\(^ {57}\) This final observation indicates that the germ cells themselves may be the primary target for the toxic action of MAA.

The incorporation of radioactivity from 2-\[^{14}\text{C}]\text{ME}\) and \[^{14}\text{C}]\text{MAA}\) into macromolecules in the maternal and conceptus compartments of pregnant mice, and the attenuation of 2-ME- and MAA-induced teratogenicity by natural substrates for the tetrahydrofolate-catalysed carbon oxidation pathways indicates that MAA may exert its teratogenic effects by acting as a false substrate in the synthesis of purine and pyrimidine nucleotides.\(^ {58-62}\) Reports that 2-ME-induced testicular degeneration in the rat is attenuated by serine, sarcosine, glycine, and acetate indicate that a similar mechanism may operate within the spermatocytes, perhaps interfering with RNA synthesis in these cells.\(^ {63}\)

1.1.3b Phthalate esters.

The diesters of phthalic acid (1,2-benzenedicarboxylic acid) are an important class of plasticisers which are used in food packaging and medical plastics. Although they are a large class of chemicals, only the \(n\)-butyl, \(n\)-pentyl, \(n\)-hexyl, and 2-ethylhexyl phthalate
diesters are known to cause testicular atrophy when administered to rats.\textsuperscript{64, 65}

The testicular lesion induced by the phthalate diesters \textit{in vivo} is characterised by seminiferous tubular atrophy, seen as exfoliation of the spermatocyte and spermatid populations, and a loss of germ cell-specific enzyme activity.\textsuperscript{64, 66 - 70} Studies with di-\textit{n}-pentyl phthalate (DPP) have shown early (within 3 h) cytological changes within the Sertoli cells, and implicate these cells as a possible locus for the toxic effects of the phthalate esters.\textsuperscript{67, 68}

Di-(2-ethylhexyl) phthalate is hydrolysed \textit{in vivo} to the monoester, mono-(2-ethylhexyl) phthalate (MEHP), the alkyl chain of which is then oxidised.\textsuperscript{70 - 72} The monoesters are considered to be the ultimate toxicants since the toxic monoesters induce degenerative changes in Sertoli-germ cell co-cultures and biochemical alterations in cultured Sertoli cells whereas the diesters, non-toxic monoesters and further oxidative metabolites of MEHP do not, and as MEHP is not significantly metabolised by cultured Sertoli cells further metabolism is unlikely to play a role in the development of the toxic lesion observed in \textit{in vitro} studies.\textsuperscript{53, 70, 73 - 75} In addition, Creasy \textit{et al} have described marked similarities between the ultrastructural changes induced in the Sertoli cells by the diesters \textit{in vivo} and the monoesters \textit{in vitro}, indicating a common aetiology.\textsuperscript{74}

Toxic monoesters stimulate the oxidation of glucose by cultured Sertoli cells, and the secretion of lactate and pyruvate into the incubation medium.\textsuperscript{56, 76, 77, 78} Complimentary results have been reported \textit{in vivo}. The administration of di-\textit{n}-butyl phthalate to rats resulted in alterations in testicular carbohydrate metabolism,\textsuperscript{79} and an increase in lactate concentration within the testes.\textsuperscript{1} At the same time, succinate dehydrogenase activity in the Sertoli cell mitochondria is inhibited.\textsuperscript{67, 78, 79} However, it is unclear as to whether these alterations in carbohydrate metabolism are fundamental or incidental to the mechanism of phthalate ester-induced testicular toxicity.

MEHP inhibits the FSH-induced stimulation of cAMP production by cultured Sertoli

\footnote{M. Fukuoka. \textit{Personal communication}.}
cells, but does not inhibit the stimulation induced by other effectors (i.e. isoproterenol, cholera toxin, forskolin). Interference either with the interaction between FSH and its receptor, or with subsequent transmembrane signal transduction processes has been proposed as a mechanism by which MEHP may exert its toxicity.\textsuperscript{61-63} Indeed, this may explain the greater sensitivity of the immature testis to the effects of the phthalate esters,\textsuperscript{64} since the immature testis has a greater dependency upon FSH than that of the adult (§1.1.2a). However, MEHP causes exfoliation of germ cells from testicular co-cultures in the absence of FSH,\textsuperscript{53, 70, 73, 74} and under these circumstances an interference by MEHP upon FSH-induced alterations in cAMP metabolism may be ruled out (although germ cells do apparently stimulate cAMP production by Sertoli cells,\textsuperscript{83} and MEHP may interfere with this paracrine interaction). Furthermore, many of the changes in homeostasis induced by MEHP in cultured Sertoli cells actually mimic the effects of increases in intracellular cAMP levels (e.g. stimulation of the secretion of lactate and pyruvate, of the oxidation of glucose, and of lipid synthesis).\textsuperscript{56, 76-78} Considering these points, it would seem that this proposed mechanism does not in itself offer a convincing explanation of phthalate toxicity in the testis.

1.1.3c Nitroaromatic compounds.

The nitrobenzenes and nitrotoluenes are important industrial intermediates in the manufacture of dyes, explosives, and polyurethane foams. They cause testicular atrophy associated with degeneration within the seminiferous epithelium, and characterised by disruption of spermatogenesis and the formation of multi-nucleate giant cells.\textsuperscript{84-89} The administration of a single dose of nitrobenzene to rats causes degeneration of the primary and secondary spermatocytes within 24 h,\textsuperscript{84} and 1,3-dinitrobenzene (DNB) causes specific degeneration and exfoliation of stage VII-XIII pachytene spermatocytes.\textsuperscript{85, 86} In addition, 2,4,6-trinitrotoluene causes degeneration and necrosis of the spermatocyte, spermatid, and spermatozoa populations in the rat.\textsuperscript{87} The Sertoli cells are thought to represent at least one locus of action for the
nitroaromatics. Experiments in rats have shown that the effects of 1,3-DNB upon the germ cells are preceded by ultrastructural changes within the Sertoli cell population,\textsuperscript{85} and that 2,4-dinitrotoluene (DNT) also induces structural changes within the Sertoli cells which are accompanied by increases in serum FSH levels,\textsuperscript{88} an indicator of Sertoli cell malfunction.\textsuperscript{89} Exposure of Sertoli-germ cell co-cultures to 1,3-DNB and toxic isomers of dinitrotoluene resulted in exfoliation of the germ cells and cytological changes within the Sertoli cell population.\textsuperscript{91,92} In addition, these compounds stimulated the secretion of both lactate and pyruvate by cultured Sertoli cells.\textsuperscript{56,92} These observations clearly indicate that biochemical and morphological changes occur within the Sertoli cell population upon exposure to toxic nitroaromatic compounds.

The mechanism(s) of nitroaromatic-induced toxicity is (are) unclear, but 1,3-DNB is metabolised by cultured Sertoli cells by a pathway which is proposed to involve redox cycling of the xenobiotic.\textsuperscript{91} The production of active oxygen species by such activity may result in oxidative stress within these cells. Cultured Sertoli cells exposed to 1,3-DNB and three toxic isomers of dinitrotoluene exhibited a decrease in the ratio of lactate to pyruvate that they secreted into the incubation medium.\textsuperscript{56,92} This suggests an increase in the oxidation state of cellular pyridine nucleotides that would be consistent with such a theory.

\subsection{1.1.3d Alkylating agents.}

Alkylating agents are compounds incorporating alkyl moieties which are, or have the potential to become, reactive towards nucleophiles and so they react with thiol groups, proteins, phosphate esters, DNA and RNA.\textsuperscript{93} Their toxicity towards cells undergoing rapid DNA synthesis has resulted in many alkylating agents being used in cancer chemotherapy.\textsuperscript{94} Spermatogenesis is a process in which the integrity of DNA, RNA and proteins are all of vital importance, and in which synthesis of these macromolecules is extensive. Consequently, it is highly susceptible to disruption by chemicals which interfere with the synthesis and/or function of these cellular components.
i) **N**-lost derivatives (nitrogen mustards and nitrosoureas). The N-lost derivatives are a group of chemicals that include a number of widely used chemotherapeutic drugs, such as mechlorethamine (mustine), 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine), chlorambucil and cyclophosphamide.

Clear degenerative changes are seen in cells undergoing premitotic and premeiotic DNA synthesis, RNA synthesis and protein synthesis, the most susceptible stage being the spermatogonia. Although many of these agents have been shown to be capable of penetrating the blood-testis barrier and reaching the spermatocytes and spermatids, the greater toxicity towards the spermatogonia appears to be related to their preferential killing of actively cycling cells.

ii) Alkane sulphonic acid derivatives. These are a series of mono- and bifunctional esters, commonly of methane and ethane sulphonic acids. n-Butane-1,4-dimethanesulphonate (busulphan) is commonly used in cancer chemotherapy.

The antifertility effects of these compounds has been extensively studied by Jackson and co-workers. Methane dimethanesulphonate, n-propane-1,3-dimethane sulphonate and busulphan cause damage to the spermatogonial stem cells in the rat, resulting in maturation depletion of the spermatozoa. Ethane-1,2-dimethane sulphonate (EDS) however differs from its homologues in that when its effects upon fertility were studied by serial mating techniques, the susceptible cell types appeared to be the spermatocytes and spermatids, an effect resembling hypophysectomy. However, later studies showed that the primary target for EDS is the Leydig cell. The delayed effects upon the germ cells are probably a result of androgen withdrawal as the testosterone-secreting Leydig cells are destroyed.

The alkane sulphonic acid monoesters (i.e. methane, ethane, and n-propane methane sulphonates) differ from their bifunctional analogues in that their principal targets are the mature spermatids and spermatozoa. The reason for this difference probably
lies in the fact that bifunctional alkylating agents can cross-link macromolecules such as DNA and thus seriously affect dividing cells. Monofunctional agents are limited to single site activity, probably killing cells by inducing irreparable mutations in their DNA.

1.1.3e Cadmium.

Cadmium causes complete haemorrhagic necrosis within the testis,\textsuperscript{2,110-112} characterised by permanent destruction of the seminiferous epithelium and reversible degeneration of interstitial tissue.\textsuperscript{2} The morphological damage to the seminiferous epithelium is preceded by damage to the endothelial cells of the testicular vasculature,\textsuperscript{2,113} resulting in tissue oedema.\textsuperscript{110-112}

Cadmium localises within the interstitium,\textsuperscript{114} and causes primary damage to the endothelium, with morphological damage and biochemical changes occurring within the internal spermatic-pampiniform plexus complex within a few hours of cadmium administration, the venules being the most susceptible to injury.\textsuperscript{2}

The degeneration of the vasculature is considered to be the primary mechanism by which cadmium exerts its effects, because although cadmium is an extremely potent toxicant towards the seminiferous epithelium \textit{in vivo} (tissue concentrations as low as 150 ng/g, approximately 1.3 \textmu M, cause diffuse haemorrhagic necrosis), degeneration of isolated tubules requires concentrations of cadmium in the incubation medium of 20 mM.\textsuperscript{115} In addition, cadmium causes testicular degeneration in scrotum-bearing animals at tissue

\textsuperscript{1} Gunn & Gould have calculated that this is equivalent to a tissue cadmium concentration of 450 mg/g.\textsuperscript{2} However, review of the original data of Kar et al,\textsuperscript{115} from which this calculation was made, indicates that it is likely to be an overestimate. In the experiments in question, rat seminiferous tubules were isolated and incubated (10 mg tissue) without shaking, in 2 ml medium containing up to 20 mM CdCl\textsubscript{2}. Only at the highest concentration of cadmium were degenerative changes noted within the tubules, reaching an acme after 60 min. At this concentration, the total amount of cadmium in the medium would be 4.5 mg, and this may be where Gunn & Gould derived their figure from (i.e. 4.5 mg cadmium/10 mg tissue). In order for this conclusion to be reached, it must be assumed that all of the cadmium in the medium was sequestered by the tissue. Although there are no reports in the literature of 100% sequestration of cadmium by testicular tissue \textit{in vitro}, the uptake in the experiments of Kar et al is likely to be very much lower.
concentrations up to 1000-fold less than those required to cause only mild focal degeneration in animals in which the testis is normally located within the abdomen. Necrosis of cryptorchid mammalian testes by cadmium indicates that the germ cells are not the primary target for toxicity.²

The administration of testosterone does not ameliorate cadmium-induced damage, and so toxicity is not a result of androgen withdrawal.² Cadmium does interfere with the utilisation of essential metals such as calcium, zinc, selenium, iron, and copper, and may share the same transport systems as some or all of these metals.¹⁰⁶ Both zinc and selenium ameliorate cadmium-induced toxicity, as do a series of calmodulin inhibitors.¹¹¹,¹¹²,¹¹⁶-¹¹⁸ These results suggest that cadmium may interfere with the activity of essential metal-dependent enzymes,¹¹⁶-¹¹⁸ although the critical target molecule(s) and the underlying mechanisms of toxicity are unclear.
1.2 THE SERTOLI CELL.

1.2.1 Functions of the Sertoli cells.

The Sertoli cells have three primary functions that allow the maintenance of an optimum environment within the seminiferous tubules, sustaining the viability of the germ cells, and promoting and controlling their maturation development into spermatozoa:

a) mechanical support of the germ cells and protection from adverse external influences,

b) provision of nutritional substrates, and

c) endocrine and paracrine regulation of testicular function.

1.2.1a The physical support of spermatogenesis.

i) Maintenance of the blood-testis barrier. Although junctions between myoid cells restrict access to the seminiferous tubules, a much more effective barrier is formed by tight intercellular junctions between neighbouring Sertoli cells at the basal portion of the seminiferous epithelium. These junctions constitute the blood-testis barrier, a highly impermeable structure that restricts access to the seminiferous epithelium to those chemicals that can diffuse across lipid membranes, and those which can act as substrates for specific uptake processes. As a consequence, all exogenous chemicals that the germ cells are exposed to must first pass through the Sertoli cells' cytoplasm.

An important consequence of the barrier is that it enables the seminiferous epithelium to be partitioned into distinct basal, intermediate, and adluminal compartments, allowing the maintenance of unique microenvironments that presumably contribute to the different needs of classes of germ cell. Spermatogonia and preleptotene spermatocytes are confined to the basal compartment, leptotene spermatocytes are found within the intermediate compartment, and more mature spermatocytes and spermatids are arranged hierarchically within the adluminal compartment (Figure 1.3). Comparison of the chemical profiles of tubular fluid and blood indicates that the Sertoli cells are capable of maintaining a
distinct microenvironment within the tubules.\textsuperscript{6, 119 - 124}

A demonstration of the importance of the blood-testis barrier to the viability of the germ cells occurs in cases of retinol (vitamin A) deficiency. Retinol serves as a carrier for glucose during the glycosylation of membrane glycoproteins, the synthesis of which is perturbed by retinol deficiency.\textsuperscript{125 - 128} Sertoli cells actively synthesise glycoproteins that are distributed to cellular structures, including the plasma membrane where they are probably involved in determining the integrity of cell-cell interactions.\textsuperscript{125, 129} If rats are fed a retinol-deficient diet after weaning, plasma retinol levels progressively fall to a nadir after 80 days. By 90 days the animals exhibit a degeneration of the blood-testis barrier, visualised as an increase in the permeability of the barrier to lanthanum, with a resultant abnormality of the tubular environment. After 100 days there is a severe disruption of spermatogenesis, including germ cell loss and degeneration.\textsuperscript{130, 131} The integrity of the barrier, and its ability to maintain a unique microenvironment within the epithelium, are therefore essential for the normal progress of spermatogenesis.

The Sertoli cells appear to play an active role in the movement of leptotene
spermatocytes across the blood-testis barrier by dissolving the tight junctions above the germ cells, introducing cytoplasmic intrusions between the germ cells and the basal lamina, and reforming the tight junctions at these new contact points. Thus the germ cells are taken into the adluminal compartment without the integrity of the barrier being breached.\textsuperscript{119}

\textit{ii) Ectoplasmonic specialisations.} Germ cells from the stage VII pachytene spermatocytes to the step 19 spermatids face subsurface structures within the Sertoli cells. These structures, termed ectoplasmonic specialisations, appear to be associations of microfilaments and endoplasmic reticulum. Their function is not fully understood, but they are thought to lend rigidity to the cell surface, providing recesses for the germ cells and facilitating their movement within the seminiferous epithelium.\textsuperscript{123, 124}

1.2.1b \textbf{The provision of essential substrates for germ cell metabolism.}

The systemic isolation of the germ cells means that they are not directly exposed to the same range of nutritional factors that other cells enjoy. In addition, the germ cells exhibit biochemical deficiencies that prevent them from carrying out 'normal' metabolic functions with the same substrates used by other cell types. Many intercellular pathways by which the Sertoli cells biochemically support the germ cells have been described or proposed. A few of these are described below by way of illustration.

\textit{i) Energy-yielding substrates.} Isolated rat germ cells utilise glucose at a considerable rate, but very little of the pyruvate that they produce is oxidised by the TCA cycle. Indeed glucose is unable to maintain ATP levels, oxygen consumption, and protein and RNA synthesis, and to promote the incorporation of \textsuperscript{32}P into ATP in germ cells.\textsuperscript{132, 143} In contrast, these cells oxidise both lactate and pyruvate, and when they are incubated in the presence of either or both of these substrates, with or without glucose, ATP levels and cellular functions are maintained.\textsuperscript{132, 140, 143, 146} However, the concentration of lactate in rat blood (1 mM),\textsuperscript{147} is
lower than that required by the cells in vitro (3-6 mM).

Cultured Sertoli cells metabolise glucose at a high rate, but only a small proportion (less than 5%) of the glucose that they utilise is oxidised in the TCA cycle. Much of the pyruvate produced by glycolysis is reduced to lactate, and these two metabolites are released from the cells into the culture medium. This may allow the Sertoli cells to support the energy demand of the germ cells, by maintaining a high lactate concentration within the intercellular milieu.

The role of lactate secreted by Sertoli cells in the maintenance of germ cell function is demonstrated by the work of Jutte and co-workers. Isolated rat germ cells incubated with glucose as the sole energy source do not incorporate significant amounts of [³H]uridine or [¹⁴C]leucine into cellular macromolecules, but such incorporation is supported by lactate. If germ cells are co-cultured with Sertoli cells, then incorporation of [³H]uridine is maintained by glucose, and the incorporation of [¹⁴C]leucine by rat germ cells cultured alone is stimulated by incubation in Sertoli cell-conditioned medium. These observations clearly indicate that the Sertoli cells support germ cell functions by regulating the supply of energy-yielding substrates, and have led many workers to conclude that one role of the Sertoli cells is to supply the germ cells with essential energy-yielding substrates.

This is further illustrated by the toxicant 5-thio-D-glucose, a glucose analogue which inhibits spermatogenesis in rats and mice. This anti-spermatogenic effect is unlikely to be as a result of a direct effect upon the germ cells themselves, because of their functional independence from glucose. However, 5-thio-D-glucose inhibits glucose metabolism by the testis, and reduces the secretion of lactate and pyruvate by cultured Sertoli cells. The anti-spermatogenic effects of 5-thio-D-glucose could therefore be a result of an indirect energy starvation of the germ cells.

ii) Intercellular GSH metabolism. Reduced glutathione (GSH) is an important cellular thiol involved in many vital aspects of cellular homeostasis, including maintenance of protein thiol
groups and detoxification of peroxides and free radicals. GSH in round spermatids is known to have an important function in the defence mechanisms against peroxides, electrophilic xenobiotics, and radiation-induced DNA damage. GSH is a tripeptide (\(N-(N\text{-}L\text{-}\gamma\text{-glutamyl-L-cysteinyl})\text{glycine}\)) which is synthesised by two conjugation reactions; the first between glutamate and cysteine, and the second between \(\gamma\text{-glutamylcysteine}\) and glycine. Rat testes have a high GSH content and actively synthesise GSH. Rat testes have a high GSH content and actively synthesise GSH.

Primary spermatocytes and round spermatids isolated from the testes of the hamster and *Xenopus laevis* have a high GSH content, but have only a limited capacity for GSH synthesis and cannot maintain these levels during culture. However, cultured hamster Sertoli cells and *Xenopus* testicular fragments actively synthesise GSH, and they are able to maintain their GSH content during culture. In addition, the GSH content of hamster and *Xenopus* germ cells are maintained when they are co-cultured with Sertoli cells, and are restored in the presence of Sertoli cells when reduced by 90% after exposure to diethylmaleate, a GSH-depleting agent.

These findings suggest that there exists an intercellular pathway for GSH metabolism within the seminiferous epithelium, in which the Sertoli cells synthesise and secrete GSH, which is then sequestered by the germ cells, allowing them to maintain homeostasis in the absence of a sufficient GSH-synthesising capacity.

**iii) Iron transport within the seminiferous epithelium.** Rat Sertoli cells have specific surface binding sites for transferrin, and double-labelled ([\(^{59}\text{Fe}\),\(^{125}\text{I}\)]) diferric transferrin is internalised by cultured rat Sertoli cells and isolated seminiferous tubules. The iron is retained, while the protein is recycled and released into the culture medium.

Cultured rat Sertoli cells have been shown to secrete a transferrin-like protein, and to release iron in a low molecular weight protein form. In addition, rat germ cells carry surface receptors that specifically bind transferrin, and internalise iron released by Sertoli
Therefore, a pathway for iron transport to the adluminal germ cells has been proposed, by which iron-bearing transferrin in the bloodstream binds to, and is then internalised by the Sertoli cells. The iron is removed, and the transferrin released back into the bloodstream. Once inside the Sertoli cells the iron is transported through the cytoplasm and, carried by a binding protein, is released into the intercellular milieu from which it is internalised by the germ cells.

iv) (Fluoro)acetate metabolism. Fluoroacetic acid is a potent cytotoxicant. It enters the TCA cycle in an analogous fashion to acetic acid, forming fluoroacetyl coenzyme A and subsequently fluorocitrate by successive condensation reactions, firstly with coenzyme A and then oxaloacetate. Fluorocitrate inhibits the activity of aconitase in the TCA cycle. Fluoroacetate is a potent inhibitor of spermatogenesis, with degeneration of the seminiferous epithelium occurring at sub-lethal doses. Fluoroacetate also inhibits pyruvate oxidation and reduces ATP levels in isolated rat seminiferous tubules but, although fluorocitrate reduces ATP levels in isolated rat germ cells, fluoroacetate itself is without effect. Further, isolated germ cells oxidise exogenous acetate at a very low rate and are seemingly unable to use it as an energy source, suggesting that germ cells cannot activate exogenous (fluoro)acetate to (fluoro)acetyl coenzyme A.

1.2.1c The cycle of Sertoli cell function.

Spermatogenesis is a cyclical process, and as the germ cell population within the epithelium changes, so too do its requirements for supporting factors. For example, the number of germ cells associated with a particular region of the epithelium is not constant (reaching a nadir at stage IX as a result of spermiation, and a maximum at stages XIII and XIV as the spermatocytes undergo the two meiotic divisions, Figure 1.1). In addition, physical alterations within the epithelium take place as leptotene spermatocytes enter and
stage 19 spermatids leave. The close association of Sertoli cell functions with germ cell development therefore requires that they are adaptable to the changing needs of the germ cell population.

i) The interaction of FSH and testosterone with the seminiferous epithelium at different stages of the cycle. Sertoli cell function is under the control of both FSH and testosterone (§1.2.2a), which have preferential stages of action during the cycle of the seminiferous epithelium (Figure 1.4). Maximal binding of FSH and activation of FSH-dependent adenylate cyclase in the Sertoli cells occurs at stages XII - V of the cycle. This is coincident with the maximal FSH-induced secretion of cAMP by isolated tubules. The maximal concentrations of testosterone in the tissue are found at stages VII and VIII, and these stages also secrete the most ABP.5,172

ii) Protein secretion. Isolated seminiferous tubules secrete at least fifteen proteins in a

Figure 1.4. Hormonal interaction with the seminiferous epithelium. FSH binding capacity (circles) and testosterone concentration (triangles) varies with the cycle of the seminiferous epithelium. Note that the two hormones interact most strongly with different stages of the cycle. Adapted from Parvinen.5
cyclical fashion, and there are two distinct peaks of protein secretion at stages VI and XII. The patterns of protein secretion at these two stages are different, and all other stages display intermediate patterns. In contrast, the pattern of cellular protein synthesis remains relatively constant throughout the cycle.\(^1_{73}\)

The secretion of transferrin by isolated seminiferous tubules is maximal at stages XIII - XIV, and then remains fairly constant until stage VI, when it falls sharply to its minimum at stage VII. This pattern is reflected by the levels of transferrin mRNA transcripts in the Sertoli cells, and correlates with fluctuations in the numbers of germ cells in the seminiferous epithelium. Germ cells from adult rats stimulate transferrin secretion by immature rat Sertoli cells in culture, and so the changing pattern of transferrin expression may represent adaptations in the provision of iron to the intercellular milieu, as the requirements of the germ cell population alter.\(^1_{73}-1_{75}\)

The stem cells and early (preleptotene) spermatocytes lie on the basal lamina, outside the permeability barrier formed by the Sertoli cells. At stages VIII and IX of the cycle the newly-formed leptotene spermatocytes are released from the basal lamina and pass across the barrier.\(^1_{19}\) This coincides with an increase in the synthesis and secretion of plasminogen activator by the Sertoli cells at stages VII and VIII,\(^5,1_{76},1_{77}\) suggesting that one role for plasminogen activator may be to allow the restructuring of the seminiferous epithelium that is required for spermatogenesis to proceed. Lacroix et al reported that testicular plasminogen activator activity increased between 40 and 60 days of age in the rat, but that it was not associated with the spermatids and spermatozoa. In addition, these authors demonstrated that plasminogen activator activity in Sertoli cell cultures was stimulated by the addition of dead \textit{Escherichia coli}. They concluded that plasminogen activator may be associated with the phagocytosis of residual bodies that are formed during spermiation at stage VIII.\(^1_{78}\)
1.2.2 Regulation of Sertoli cell function.

The function of Sertoli cells in the immature rat testis is under complex endocrine and paracrine control and it is by these mechanisms that the initiation and maintenance of spermatogenesis is regulated.

1.2.2a Endocrine control of Sertoli cell function.

Sertoli cells are influenced by a wide range of hormones (Table 1.2), but only two of these, FSH and testosterone, have been widely studied.

i) FSH. FSH binds to tissue- and cell-specific, high-affinity, low-capacity receptors upon the surface of the Sertoli cell plasma membrane. The Sertoli cell is therefore considered to be the principal, and perhaps sole, target for FSH in the male, and all influences of this hormone upon spermatogenesis are probably mediated by these cells.

The effects of FSH on the Sertoli cells are mediated by a guanylate nucleotide-binding (G) protein which modulates adenylate cyclase activity. FSH enhances the replacement of GDP by GTP at the regulatory site of the G protein, and the 'activated' G protein then stimulates the activity of adenylate cyclase, which catalyses the conversion of ATP to cAMP. In addition, the activity of cAMP-dependent phosphodiesterase (3',5'-cyclic nucleotide 5'-nucleotidase), which hydrolyses cAMP to 5'-AMP, is inhibited. The increase in cAMP synthesis and decrease in its hydrolysis together result in an elevation of intracellular levels of cAMP, which acts as the secondary messenger in the hormonal signalling system (Figure 1.5).

The hormone signal is attenuated by the hydrolysis of the GTP bound to the regulatory site of the G protein to GDP, whilst the secondary messenger signal is attenuated by a delayed increase in the activity of cAMP-dependent phosphodiesterase (PDE). These effects are unique to the adenylate signalling system, since FSH does not alter the activities of either guanylate cyclase or cGMP-dependent PDE.
Table 1.2. Hormonal regulation of Sertoli cell function. This table gives an indication of the range of Sertoli cell functions that are under hormonal influence. It is not intended to be an exhaustive list. Abbreviations: FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone; T₃, triiodothyronine; †, stimulation of activity; ↓, inhibition of activity.

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<thead>
<tr>
<th>Hormone</th>
<th>Functional Parameter</th>
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The mechanism of action of FSH. [1] FSH binds to its receptor (R) on the surface of the Sertoli cell. [2] The FSH-receptor complex moves within the plasma membrane to interact with a regulatory G protein (G) causing the displacement of GDP at the regulatory site in favour of GTP [3]. [4] The activated G protein stimulates the activity of adenylate cyclase (AC) and the GTP is hydrolysed to GDP. [5] Adenylate cyclase converts ATP to cAMP which stimulates the activity of cAMP-dependent proteins within the cell [6].

The role of cAMP in mediating FSH-regulation of Sertoli cell function has been confirmed in two ways. The inhibition of cAMP PDE, which catalyses the hydrolysis of cAMP, enhances the effects of FSH. In addition, the effects of FSH are mimicked by lipid-soluble derivatives of cAMP (e.g. dbcAMP and 8-bromo cAMP) and by agents that directly stimulate the activity of adenylate cyclase (e.g. forskolin and cholera toxin).

The rapid increase in the concentration of intracellular cAMP results in the activation of cAMP-dependent protein kinase (ATP: protein phosphotransferase), which is thought to provide the link between the hormonal signal and subsequent cellular responses.

Cultured Sertoli cells incubated in the presence of FSH undergo morphological alterations. The cells form a flattened, confluent monolayer in culture, but upon exposure to FSH they become rounded, with filamentous cytoplasmic protrusions to anchorage sites on the support substrate giving them a 'spiky' appearance. These cytoskeletal...
alterations are thought to play a role in the exocytotic activity of the cells. The energy required may be derived from endogenous glycogen stores, since FSH stimulates the activity of glycogen phosphorylase, an enzyme that is closely associated with the cytoskeleton.

FSH stimulates RNA polymerase activity in cultured Sertoli cells, resulting in an increase in RNA synthesis. As a consequence, the synthesis and secretion of a broad spectrum of proteins are stimulated, including ABP, plasminogen activator, and inhibin.

FSH has a profound effect upon glucose metabolism in cultured immature Sertoli cells. Uptake of 3-O-methyl-D-glucose, a non-metabolisable glucose analogue, is stimulated by FSH and dbcAMP, and this is reflected as an increase in glucose consumption, resulting in increases in glycolysis, glucose oxidation, and the secretion of lactate and pyruvate. The stimulation of glucose metabolism seems to be unspecific, since dbcAMP also markedly stimulates inositol secretion by cultured Sertoli cells, and FSH stimulates the incorporation of radioactivity from D-[U-14C]glucose into lipids.

Testosterone is aromatised by the Sertoli cells to 17β-oestradiol ((17β)-oestra-1,3,5(10)-triene-3,17-diol), a conversion which is stimulated by FSH. However, aromatase activity in vivo decreases as the germ cell population increases, and the germ cells release factor(s) that inhibit 17β-oestradiol secretion by cultured Sertoli cells. This may reflect a mechanism by which FSH initiates spermatogenesis, but which is no longer required once the process is underway.

**ii) Testosterone.** Testosterone enters the Sertoli cells, where it binds to a specific cytosolic receptor. The testosterone-receptor complex is translocated to the nucleus where it stimulates the activity of RNA polymerase. The result is an increase in both RNA and protein synthesis.

One of the proteins whose synthesis is stimulated by testosterone is ABP, which is
distinct from the cytosolic receptor and transports androgens into the lumen.\textsuperscript{9, 27}

Morris \textit{et al} have also reported that testosterone and androstenedione inhibit the
secretion of inhibin, and its stimulation by FSH, from cultured Sertoli cells.\textsuperscript{23}

\textit{iii) Other hormones.} Sertoli cells have been shown to have receptors for, and/or respond to,
a wide range of other hormones. Functional alterations induced by other hormones include
insulin-induced stimulation of lactate and pyruvate secretion,\textsuperscript{149, 185} transferrin secretion,\textsuperscript{204} and
\textsuperscript{D}-\textsuperscript{[U\textsuperscript{14}C]}glucose incorporation into lipids;\textsuperscript{185} glucagon-induced stimulation of cAMP
production;\textsuperscript{180, 219} and thyroid-stimulating hormone-induced alterations in morphology,\textsuperscript{205, 206}
and increases in the secretion of ABP, plasminogen activator, and lactate.\textsuperscript{197, 206, 220} In
addition, cultured Sertoli cells have been shown to contain nuclear triiodothyronine (T\textsubscript{3})
receptors, and to exhibit T\textsubscript{3} responsiveness.\textsuperscript{221, 222} However, the physiological relevance of
these reactions to hormonal stimuli, if any, remains unclear at present.

The question of hormonal regulation of Sertoli cell function in culture should be
approached and examined with caution. Cultured Sertoli cells have \p-adrenergic receptors,
and exhibit sensitivity to isoproterenol, a \p-adrenergic receptor agonist which stimulates
adenylate cyclase activity, androgen aromatisation, protein phosphorylation, and lactate and
pyruvate secretion, and alters cellular morphology.\textsuperscript{182, 188, 199, 219, 223} However, freshly isolated
seminiferous tubules and Sertoli cells show negligible \p-adrenergic receptor content. In
addition, the \p-adrenergic receptor content of cultured Sertoli cells increases with time, and
this increase can be blocked by inhibition of protein synthesis. Therefore, the \p-adrenergic
responsiveness of cultured Sertoli cells appears to be an artifact of culture.\textsuperscript{223} Similar
observations have been reported concerning acquired \p-adrenergic responsiveness by
cultured hepatocytes, and acquired adenosine responsiveness by cultured Leydig cells.\textsuperscript{224 - 226}

\textit{1.2.2b Paracrine control of Sertoli cell function.}

As described above (§1.2.2a), the functional characteristics of the Sertoli cell are
subject to alteration under the influence of the hormonal activity of the hypothalamal-pituitary-testicular axis. Pituitary hormones regulate the Sertoli cells both directly (FSH) and indirectly (LH).

However, evidence suggests that the Sertoli cell is also directly subject to factors released from the germ cells, peritubular cells, and Leydig cells (Table 1.3).

i) Control by germ cells. Certain changes in Sertoli cell functional parameters correlate temporally with changes in the germ cell population as the testis matures. For example, aromatase activity in Sertoli cells falls rapidly during sexual maturation in the rat, resulting in a reduction in the conversion of testosterone to 17β-oestradiol, whereas ABP secretion increases. In addition, the removal of germ cells from the mature rat testis in vivo, by the administration of specific toxicants or by irradiation, also causes marked alterations in Sertoli cell functional parameters.

One possible explanation for these observations is that the germ cells directly affect

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Functional Parameter</th>
<th>Effect</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Germ cell</td>
<td>Adenylate cyclase activity</td>
<td>↑</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>ABP production</td>
<td>↑</td>
<td>216-218,227-229,223</td>
</tr>
<tr>
<td></td>
<td>Transferrin production</td>
<td>↑</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>Aromatase activity</td>
<td>↓</td>
<td>214,215</td>
</tr>
<tr>
<td>Peritubular cell</td>
<td>ABP production</td>
<td>↑</td>
<td>179,234-236</td>
</tr>
<tr>
<td></td>
<td>Transferrin production</td>
<td>↑</td>
<td>204,234</td>
</tr>
<tr>
<td></td>
<td>Aromatase activity</td>
<td>↓</td>
<td>179</td>
</tr>
<tr>
<td>Leydig cell</td>
<td>Adenylate cyclase activity</td>
<td>↓</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>ABP secretion</td>
<td>↓</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>Inhibin secretion</td>
<td>↓</td>
<td>22,23</td>
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</table>
the activity of the Sertoli cells, and this is corroborated by data derived from in vitro experiments.

Germ cells and germ cell-conditioned media have been shown to stimulate the secretion of transferrin and ABP by cultured rat Sertoli cells, and to inhibit the secretion of 17β-oestradiol. In addition, Sertoli cells isolated from older rats secrete more transferrin and ABP than those from less mature animals. This suggests that altered function in vivo may partly result from alterations in the germ cell population, and that the germ cells may directly influence Sertoli cell function in accordance with their requirements.

Germ cells have been shown to stimulate adenylate cyclase activity in cultured Sertoli cells. In addition, ABP secretion and FSH-induced stimulation of secretion by Sertoli cells isolated from 20 day old rats are also elevated, an effect which is mediated by protein factor(s) released from the germ cells when cultured in the absence of Sertoli cells. Stimulation is greater at higher germ cell viabilities, and so is not due to non-specific lysis products, but to products that are specifically synthesised and secreted by the germ cells. However, germ cells appear to antagonise the effects of FSH upon Sertoli cells isolated from older (45 day) rats, suggesting that the germ cells may play a role in the age-dependent refractoriness of the Sertoli cells towards FSH.

The influence of the germ cells upon the Sertoli cells is also age-related as regards the sensitivity of the Sertoli cells towards different subpopulations of germ cells. Whereas both spermatocytes and spermatids stimulate ABP secretion and inhibit 17β-oestradiol secretion by Sertoli cells from 20 and 45 day old rats, only spermatocytes have this effect on those from 10 day old rats. This may reflect a broadening spectrum of effectors that the Sertoli cells become susceptible to as the germ cell population increases and differentiates.

The influence of germ cells upon the activity of the Sertoli cells may constitute one mechanism by which Sertoli cell functions adapt to the changing germ cell population (§1.2.1d).
ii) Control by peritubular cells. Peritubular cells contain high-affinity, androgen-specific receptors and, under the influence of testosterone and 5α-dihydrotestosterone, release protein factor(s), termed P-Mod-S, that modulate(s) Sertoli cell function. P-Mod-S stimulates ABP and transferrin production, and inhibits 17β-oestradiol secretion by cultured Sertoli cells. Sertoli cell-modulating factors are not released from cultured footpad fibroblasts in the presence of androgens, and so P-Mod-S is specific for the interaction between peritubular cells and Sertoli cells.\(^\text{11, 179, 234}\)

Conditioned medium from peritubular cells incubated in the absence of androgens has no effect upon ABP secretion, androgen aromatisation, or transferrin secretion by cultured Sertoli cells.\(^\text{179, 204, 234-236}\) However, when Sertoli cells and peritubular cells are cultured together in the absence of androgens, the secretion of ABP and transferrin are stimulated,\(^\text{204, 235, 236}\) suggesting that the peritubular cells may have some other role in controlling Sertoli cell function. In addition, if isolated Sertoli cells are co-cultured with peritubular cells that have been pre-labelled with \[^{3}H\]uridine, then radioactivity is found associated with those Sertoli cells that lie adjacent to the peritubular cells, but not with more distal Sertoli cells. Further, the association of vesicles with the plasma membranes of adjacent Sertoli and peritubular cells, and the insensitivity of the transfer of radiolabelled RNA or RNA precursor molecules to RNase, indicate that intercellular transfer of metabolites between the peritubular and Sertoli cells occurs by mechanisms other than secretion.\(^\text{236}\)

iii) Control by Leydig cells. The effects of testosterone have been discussed above (§1.2.2aii), but other regulatory pathways between Leydig cells and Sertoli cells have been proposed.

The Sertoli cells have high-affinity, low-capacity, specific opiate-binding sites. Exposure of cultured Sertoli cells to β-endorphin reduces the secretion of ABP and inhibin, and antagonises the stimulation of adenylate cyclase activity and inhibin synthesis induced by FSH.\(^\text{22, 237}\) The lack of effect of β-endorphin upon the stimulation of adenylate cyclase

- 34 -
activity and inhibin synthesis by forskolin, which stimulates adenylate cyclase directly, suggests that the opioid peptide exerts its influence by inhibiting FSH receptor coupling to adenylate cyclase.

Leydig cells contain both β-endorphin and its precursor peptide, proopiomelanocortin. In addition, the levels of proopiomelanocortin mRNA are stimulated by gonadotropins, and this raises the possibility that the Leydig cells regulate Sertoli cell function by a LH-sensitive mechanism other than testosterone secretion.

**Adenosine.** Adenosine has been implicated in the mediation of paracrine interactions between cells in many tissues. Adenosine receptors have been described in rat testes, located on the surface of both germ and Sertoli cells, but they are not associated with interstitial cells.

The adenosine receptors in the testis are of the A<sub>i</sub> (inhibitory) type. Activation of A<sub>i</sub> receptors results in attenuation of adenylate cyclase activity, an effect which is mediated by an inhibitory G protein. As a consequence, adenosine A<sub>i</sub> receptor agonists antagonise the effects of FSH and other hormones that stimulate adenylate cyclase activity. In cultured rat Sertoli cells, adenosine reduces FSH-induced cAMP accumulation and androgen aromatisation, and <sup>N</sup>-<sub>6</sub>-(2-phenyl-<i>iso</i>-propyl) adenosine (PIA), a non-metabolisable A<sub>i</sub> agonist, inhibits the FSH-dependent stimulation of cAMP accumulation, androgen aromatisation, and inhibin secretion. In addition, both PIA and another A<sub>i</sub> agonist, 2-chloroadenosine, inhibit glucagon-induced cAMP accumulation.

PIA also reduces the stimulation of androgen aromatisation induced by forskolin and cholera toxin, but has no effect upon the stimulation of androgen aromatisation and inhibin secretion induced by dbcAMP, confirming that the effects of adenosine A<sub>i</sub> agonists upon cultured Sertoli cells are mediated solely by an inhibition of adenylate cyclase activity.

Cultured cells may acquire functional responsiveness to effectors that they do not normally possess in vivo. However, the responsiveness of Sertoli cells to adenosine in
vitro is not likely to be an artifact of culture since PIA also antagonises the FSH-induced stimulation of cAMP accumulation in intact and dispersed testes. This indicates that functional A receptors are present on Sertoli cells in vivo, although the source(s) of adenosine within the testis, and its role in modulating testicular function are as yet unknown.
1.3 CREATINE.

Creatine \((N\text{-}(\text{aminoiminomethyl})\text{-}N\text{-}methylglycine)\) is a widely distributed disubstituted guanidine derivative. The structures of creatine and two other important and metabolically related guanidine derivatives, guanidinoacetic acid (GAA, \(N\text{-}(\text{aminoiminomethyl})\) glycine) and arginine, are shown in Figure 1.6A.

The basic and acidic groups of creatine have \(pK_a\) values of 14.3 and 2.63 respectively,\(^{245}\) and under physiological conditions creatine exists as a zwitterion, with the protonated basic group being further stabilised by resonance (Figure 1.6B).

1.3.1 Creatine metabolism.

In the rat creatine is found predominantly in muscle, brain, and testis, with comparatively little in the liver and kidneys.\(^{246-252}\) In those tissues in which it is utilised, a proportion of creatine is found as a phosphorylated derivative, \(N\text{-}phosphorylcreatine (PCr, §1.3.1bi)\). Since creatine and PCr are continually converted to the anhydride creatinine (§1.3.1biii),\(^{253-257}\) which is excreted in the urine, the creatine pools of these tissues must be constantly replenished. This is accomplished both by dietary intake and by \textit{de novo} synthesis.

![Figure 1.6. Creatine. A) The structures of creatine, GAA, and arginine. B) Resonance structures of creatine.](image-url)
1.3.1a The biosynthesis of creatine.

i) The pathway for creatine synthesis. Creatine is synthesised in two stages, the amidine group from arginine is transferred to glycine by glycine amidinotransferase (L-arginine: glycine amidinotransferase, GAT) to produce GAA (Equation 1.1), which is then methylated by guanidinoacetate methyltransferase (S-adenosyl-L-methionine: guanidinoacetate N-methyltransferase, GMT) to creatine with S-adenosylmethionine acting as the methyl group donor (Equation 1.2).

\[
\begin{align*}
\text{GAT} & : \quad \text{arginine} + \text{glycine} \xrightarrow{\text{GAT}} \text{GAA} + \text{ornithine} \\
\text{GMT} & : \quad \text{GAA} + S\text{-adenosylmethionine} \xrightarrow{\text{GMT}} \text{creatine} + S\text{-adenosylhomocysteine}
\end{align*}
\]

In man and the chick these two reactions are both carried out in the liver, but in laboratory animals, including the rat, the formation of GAA occurs primarily in the pancreas and kidney.

GAT is a cytosolic and/or mitochondrial enzyme that catalyses the reversible transfer of the amidine group (-C[=NH]NH₂) from arginine to glycine, producing GAA and ornithine. The reversible nature of this reaction was recognised by the inhibitory effect of ornithine upon the enzyme, and from the incorporation of radioactivity from \(\text{[guanidino-}^{14}\text{C}]\text{GAA}\) into the amidine group of arginine. GAT appears to have two substrate binding sites, one for the donor molecule and the other for the acceptor, and catalyses the transfer of the amidine group by forming an 'active urea' intermediate with a cysteine thiol group at the active site (Figure 1.7). The amidine group is then transferred to the acceptor molecule by the reverse reaction.

GMT is a cytosolic enzyme that catalyses the methylation of GAA to creatine. This reaction is essentially irreversible, and therefore represents the committed step in creatine synthesis.
Figure 1.7. The mechanism of GAT action. The amidine group from the donor molecule is transferred to a thiol group at the active site of the enzyme (Enz) to form an 'active urea' intermediate. The reverse reaction requires the acceptor molecule to be in the uncharged state.\textsuperscript{253}

\[ R\text{C}=\text{NH}_{2} + \text{Enz-SH} \rightleftharpoons \text{Enz-S-C} + \text{RNH}_{2} \]

\( ii) \) The regulation of creatine synthesis. The three precursors of creatine (i.e. L-arginine, glycine, and L-methionine) are all essential substrates for the maintenance of cellular homeostasis and protein synthesis, and because the synthesis of creatine is essentially irreversible its control is an important biochemical consideration. The regulation of creatine synthesis has been extensively studied in the chick embryo and chick liver, this model being chosen because it has both enzymes for creatine biosynthesis, and the chick embryo can further be considered a closed system which originates with no creatine content at all.\textsuperscript{253}

The formation of GAA is the rate-limiting step in the synthesis of creatine since both L-arginine and glycine, and particularly the former, are found to be rate-limiting precursors, whilst L-methionine is not. Furthermore, GAA stimulates creatine synthesis more effectively than either L-arginine or glycine.\textsuperscript{253}

The activity of GAT in the chick embryo liver decreases with increasing creatine concentration and is also reduced by GAA, L-arginine, glycine (but only in the presence of L-arginine), and by the creatine analogue \( \text{N-acetimidoylsarcosine} \) (substitution with \(-\text{C}[=\text{NH}]\text{CH}_{3}\) for \(-\text{C}[=\text{NH}]\text{NH}_{2}\)). The equivalent GAA and L-arginine analogues are without effect, suggesting that it is creatine itself, and not one of its precursors, that is the ultimate active agent in suppressing the activity of GAT. This is further supported by evidence that L-ethionine, which as S-adenosyl-L-ethionine acts as a competitive inhibitor of GMT, antagonises the GAA-induced reduction in GAT activity. In addition, \( \text{N-acetimidoylsarcosine} \) is not a substrate for phosphorylation, indicating that it is not \( \text{PCr} \) that reduces GAT activity.\textsuperscript{253}
Although GAT is directly inhibited by ornithine in vitro, and this may also occur in vivo, creatine does not act as a direct inhibitor of GAT. Rather, it represses activity in a manner similar to the protein synthesis inhibitor cycloheximide, suggesting that the inhibitory effect of creatine upon GAT activity is by the repression of synthesis of GAT protein. When the GAT-repressed chicks hatch, liver GAT activity returns to control values as long as they are maintained on a creatine-free complete diet, derepression representing synthesis of new GAT protein.

**iii) Nitrogen metabolism and its relation to creatine synthesis.** The carbon skeletons of surplus amino acids may be used as energy-yielding substrates after their constituent nitrogen atoms have been removed. In most cases the 2-amino group is removed by transamination with 2-oxoglutarate in a reaction catalysed by aminotransferases (AT)

\[
\text{2-amino acid} + \text{2-oxoglutarate} \xrightarrow{\text{AT}} \text{2-oxo acid} + \text{L-glutamate}
\]

Equation 1.3

Glutamine is directly hydrolysed to glutamate and ammonia by glutaminase (L-glutamine amidohydrolase).

The carbon skeleton of glutamate can enter the TCA cycle as 2-oxoglutarate after it has been oxidatively deaminated by mitochondrial glutamate dehydrogenase (L-glutamate: NAD(P)^+ oxidoreductase (deaminating), GluDH):

\[
\text{L-glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{GluDH}} \text{2-oxoglutarate} + \text{NADH} + \text{NH}_4^+
\]

Equation 1.4

ATP, GTP and NADH are allosteric inhibitors of GluDH, whereas ADP and GDP are allosteric activators. Thus GluDH activity is regulated by the energy charge of the cell.

Amino acid metabolism therefore results in the production of ammonia. Ammonia may be used in the biosynthesis of new amino acid molecules, being incorporated by the
reversal of the reaction in Equation 1.4. However, excess ammonia is toxic, particularly to the brain, in part due to the removal of 2-oxoglutarate from the TCA cycle.\textsuperscript{259, 261}

In mammals ammonia is detoxified by two important pathways, by incorporation into urea and into the amide group of glutamine.\textsuperscript{259, 261} Ureagenesis occurs predominantly in the liver, with the glutamine pathway for detoxification occurring in other tissues. The synthesis of urea follows a cyclical pathway that is partitioned between the mitochondria and the cytosol.

Ammonia is condensed with carbon dioxide and a high-energy phosphoryl group in the mitochondria by carbamoyl phosphate synthetase in a complex reaction that uses two molecules of ATP, making it essentially irreversible, forming carbamoyl phosphate. The carbamoyl group is transferred to ornithine by ornithine transcarbamoylase to produce citrulline which passes into the cytosol before condensing with aspartate (formed from the transamination of oxaloacetate with an amino acid) to produce arginosuccinate. This reaction is catalysed by arginosuccinate synthetase and driven by the hydrolysis of ATP to AMP and pyrophosphate and the subsequent hydrolysis of the pyrophosphate. The cleavage of arginosuccinate by arginosuccinase to arginine and fumarate (which is converted to oxaloacetate in the TCA cycle), and hydrolysis of arginine by arginase to urea and ornithine completes the cycle. The net balance for urea synthesis is summarised in Equation 1.5.

\[
\text{NH}_2 + \text{CO}_2 + 3\text{ATP} + \text{amino acid} + 2\text{H}_2\text{O} \rightarrow \text{urea} + 2\text{ADP} + \text{AMP} + 4\text{P}_i + 2\text{-oxo acid} \quad \text{Equation 1.5}
\]

Glutamine is synthesised by amination of glutamate with ammonia by the enzyme glutamine synthetase (L-glutamate: ammonia ligase (ADP-forming), GlnSyn):

\[
\text{L-glutamate} + \text{NH}_2 + \text{ATP} \xrightarrow{\text{GlnSyn}} \text{L-glutamine} + \text{ADP} + \text{P}_i \quad \text{Equation 1.6}
\]

The synthesis of glutamine uses one high-energy bond for each ammonium ion bound.
(Equation 1.6), whereas ureagenesis uses four such bonds (Equation 1.5). Therefore ammonia detoxification by incorporation into glutamine is more energy efficient and is the path of choice for most extrahepatic sites of ammonia production. If $^{15}\text{NH}_4^+$ is administered to rats it is found that glutamine is more extensively labelled than urea.\textsuperscript{262}

GAA is synthesised in an analogous fashion to urea in that the amidine group of arginine is removed (by transamidination and hydrolysis respectively), producing ornithine as the other reaction product. However, if rats are fed $^{15}\text{NH}_4^+$ in a normal diet the incorporation of $^{15}\text{N}$ into creatine is very low in comparison to urea, but it is increased substantially if the animals are kept on a low nitrogen diet.\textsuperscript{262-265} The low incorporation of $^{15}\text{N}$ from $^{15}\text{NH}_4^+$ into creatine in comparison to urea probably represents the compartmentalisation of GAA and urea synthesis between the organs of the rat. Both ureagenesis and GAA synthesis require arginine, which is formed in the liver as part of the urea cycle whereas GAA synthesis occurs predominantly in the kidney and pancreas.\textsuperscript{253}

1.3.1b The further metabolism of creatine.

The only recognised metabolic fates of creatine are a reversible phosphorylation reaction, and cyclisation to the anhydride creatinine.\textsuperscript{253-255}

i) $N$-Phosphorylcreatine (PCr). The presence of an acid-labile phosphorous compound in vertebrate skeletal muscle was first reported over 60 years ago by Eggleton and Eggleton and Fiske and Subbarow. The latter authors further isolated and purified the compound, and showed it to contain phosphoric acid and creatine in a 1:1 molar ratio. They also concluded that the compound had the structure given in Figure 1.\textsuperscript{266}

The term ‘phosphagen’ is commonly used to describe the class of naturally-occurring phosphorylated guanidine derivatives. They are compounds that contain a high-energy phosphorus-nitrogen bond (as opposed to the high-energy phosphorus-oxygen bonds of ATP, pyrophosphate, carbamoyl phosphate, phosphorylated sugars etc.)
The phosphorylation of creatine to PCr is reversible and is catalysed by the enzyme creatine phosphorlyltransferase (CPT, ATP: creatine N-phosphorlyltransferase, EC 2.7.3.2), with ATP (ADP) acting as the phosphoryl group donor (acceptor):

\[
\text{creatinine} + (\text{ATP-Mg})^2- \xrightarrow{\text{CPT}} \text{PCr} + (\text{ADP-Mg})^- \quad \text{Equation 1.7}
\]

Magnesium is required to obtain the optimal adenosine nucleotide conformation within the active site of the enzyme.\(^{266, 267}\)

CPT is distributed throughout the cell, localised both at the mitochondria and sites of energy utilisation (e.g. plasma membrane, sarcoplasmic reticulum, myofibrils). Non-mitochondrial CPT isoenzymes are homogenous or heterogenous dimers.\(^{268 - 271}\) The two different subunits are termed M (skeletal muscle-specific) and B (brain-specific), and are distinct from each other as regards their amino acid composition and sequence, electrophoretic mobility, and immunological characteristics.\(^{572}\) Thus CPT in skeletal muscle has the structure MM, that in nervous tissue BB, and in cardiac muscle MB.\(^{271}\) These isoenzymes favour the reverse reaction in Equation 1.7 (i.e. the formation of ATP).\(^{253, 273}\)

Mitochondrial CPT isoenzymes are dimeric or oligomeric structures that are derived from a unique subunit (termed M) that is distinct from both the M and B subunits.\(^{274 - 282}\) Evidence suggests that like non-mitochondrial isoenzymes, different tissues may contain different mitochondrial CPT isoenzymes.\(^{280, 281}\) Unlike the non-mitochondrial isoenzymes,
mitochondrial CPT favours the forward reaction shown in Equation 1.7 (i.e. production of PCr). Mitochondrial CPT is located on the outer surface of the inner mitochondrial membrane. When mitochondria are incubated in the presence of creatine and ADP, oxygen consumption is stimulated and PCr is produced. These observations have led to the proposal that mitochondrial ATPase, ATP/ADP translocase, and CPT may form a loose complex within the inner mitochondrial membrane allowing CPT direct access to newly-synthesised ATP.

Like creatine, PCr is extensively distributed throughout the body, although the extent of phosphorylation within these tissues differs widely.

ii) Creatinine is a waste end product of creatine metabolism. Creatinine (2-imino-1-methyl-4-imidazolidinone) is a cyclic compound and the anhydride of creatine. Its structure is given in Figure 1.9.

Bloch and co-workers observed that after [15N]creatine was fed to rats [15N]creatinine was excreted in the urine. The daily rate of creatinine excretion was calculated to approximate that of creatine synthesis.

Although creatine can spontaneously dehydrate to creatinine, PCr can undergo this reaction at a rate which is 3-4 times faster, probably via an N-phosphorylcreatinine intermediate. Since the majority of the body's PCr pool is localised within the

![Figure 1.9. Creatinine. The structure of creatinine.](image)
skeletal muscle, creatinine excretion is considered to be related to muscle mass and to be fairly constant.\textsuperscript{255, 260, 290 - 292} However, creatinine excretion does show a wide intrasubject variation, and changes greatly with trauma, infection, and malnutrition.\textsuperscript{293 - 296}

Creatinine is generally considered to be largely metabolically inactive and to represent an end-point in creatine metabolism,\textsuperscript{288} although it may serve as a urinary antioxidant by chelating metal ions which in the free form may catalyse the generation of free radical species.\textsuperscript{297}

1.3.2 The uptake of creatine by target tissues.

Because the processes of creatine synthesis and utilisation are generally confined to different organs,\textsuperscript{253} target cells must have a system for the uptake of creatine from the bloodstream. Such systems have been described and characterised in a variety of tissues and cell types including skeletal muscle, smooth muscle, fibroblasts, macrophages, monocytes, erythrocytes, brain, and adipose tissue.\textsuperscript{298 - 306}

Uptake of creatine into its target cells and tissues is generally described as a two-component system consisting of a high affinity active process and a component of diffusion.\textsuperscript{298, 299, 303, 304} The affinity of the active uptake systems (\(k_m = 20-60 \mu M\))\textsuperscript{298, 299, 302} is within the normal range of reported serum creatine concentrations,\textsuperscript{292, 307 - 310} and is therefore compatible with an efficient uptake of creatine from the blood by these tissues.

Creatine is taken up into its target tissues by a specific energy- and sodium-dependent carrier-mediated process. The specificity of uptake is amply demonstrated by the fact that uptake into a number of tissues is inhibited by other guanidine derivatives, particularly 3-guanidinopropionic acid, suggesting that uptake maybe specified by the amidine moiety. Creatine uptake is not substantially inhibited by PCr, creatinine, or amino acids.\textsuperscript{298 - 300, 302, 303}

Creatine sequestration is inhibited both at low temperatures and by inhibitors of energy metabolism,\textsuperscript{301, 305} and is therefore dependent upon the production of ATP within the
The sodium-dependence of creatine uptake has also been extensively reported. Transport is inhibited both by low concentrations of sodium in the extracellular medium, and by the inhibitor of Na\(^+\)/K\(^+\)-ATPase activity, ouabain.\(^{298,299,302,305}\) If sodium is replaced in the medium by choline, potassium or sucrose, or if the maintenance of the plasmalemmal sodium gradient is compromised by inhibition of the Na\(^+\)/K\(^+\)-ATPase transport protein, then the active transport of creatine ceases. Loike et al suggested that creatine flux is strongly influenced by the relative magnitude and polarity of the sodium gradient, since creatine efflux from macrophages was increased if cells were incubated in the absence of sodium or in the presence of ouabain.\(^{302}\) However, these workers were unable to conclude as to whether these observations were as a result of the activity of a sodium-creatine transport complex, or simply by an electrochemical sodium gradient providing the energy for transport.

Treatment of erythrocytes with the proteolytic enzyme pronase reduces the uptake of creatine into these cells by 38%,\(^{303}\) whilst n-hexanol and phloretin, which stimulate the transfer of components by the lipid phase of the plasma membrane and inhibit the transfer of components that are taken into the cell by carrier proteins,\(^{311}\) reduce creatine sequestration by 20% and 80% respectively.\(^{303}\) Moreover, extracellular creatine causes down-regulation of creatine transport into cultured rat and human skeletal muscle cells, by a mechanism that is mediated by a protein factor. It is most likely that this protein factor interacts with another protein structure, presumably the creatine carrier, at the plasma membrane.\(^{299}\)

A mechanism for the active transport of creatine has been proposed by which the electrochemical gradient produced by sodium partition across the plasma membrane is used to drive the substrate-carrier complex from the external side of the membrane to the internal side.\(^{312}\) Creatine and one or more sodium ions combine with the protein carrier, and the creatine-sodium-carrier complex then crosses the plasma membrane as sodium passes down its electrochemical gradient (Figure 1.10). Sodium is then returned to the external side of the membrane, in exchange for potassium, by Na\(^+\)/K\(^+\)-ATPase. This mechanism accounts for
Figure 1.10. Proposed mechanism for the active uptake of creatine into target tissues. [1] Creatine (Cr) and one or more sodium ions bind to the creatine carrier protein molecule (C) on the extracellular surface of the plasma membrane. [2] The creatine-sodium-carrier complex moves across the plasma membrane as sodium passes down its electrochemical gradient. [3] Creatine and sodium dissociate from the carrier protein and the sodium is removed from the cell in exchange for potassium by Na⁺/K⁺-ATPase.
the dependence of uptake on both extracellular sodium and ATP production, and for the inhibitory effects of ouabain.

1.3.3 The role of creatine and PCr in target tissues.

PCr was originally thought to represent an energy reservoir, storing high-energy phosphoryl groups that could be used to rephosphorylate ADP during periods of energy utilisation.

This theory has since been superseded by that of a creatine-PCr shuttle. In this model creatine is phosphorylated at the mitochondria and carries its high-energy phosphoryl group to sites of energy utilisation where it is used to rephosphorylate ADP to ATP. The creatine thus formed then returns to the mitochondria. This system would allow distinct advantages over the simple diffusion of ATP:

a) energy supply can be specifically directed to where it is most needed since only those sites associated with CPT can utilise PCr,

b) the diffusion coefficients of creatine and PCr are almost twice those of ADP and ATP. Because diffusion is maintained by concentration gradients, then the concentration gradient required to achieve a given energy level at a distal site of utilisation will be smaller for PCr than for ATP. Since such a concentration gradient represents 'wasted energy' (i.e. high-energy phosphoryl groups used solely to maintain such a gradient), then PCr represents a more efficient energy transduction mechanism than ATP. Similarly, the concentration of ADP at distal sites of energy utilisation (i.e. sites of ADP production) can be kept relatively low. This is an important consideration since ADP can inhibit ATPase activity.

c) ADP produced from the phosphorylation of creatine at the mitochondria is immediately returned to the sites of ATP synthesis, enabling continual stimulation of energy production (respiratory control).

d) the availability of a PCr reservoir would provide a buffer against anoxia that would

- 48 -
not be available to a cell that relied solely upon the presence of limited adenine nucleotide reserves as energy supply.\textsuperscript{315}

The creatine-PCr shuttle has been most extensively studied in the spermatozoa of the purple sea urchin, \textit{Strongylocentrotus purpuratus}. Distinct CPT isoenzymes associated with the mitochondria and flagellae of these cells have been described. The mitochondrial isoenzyme favours the phosphorylation of creatine, whilst the flagellar isoenzyme favours the phosphorylation of ADP.\textsuperscript{273, 321, 322}

Flagellar CPT is associated with the microtubular network and is thought by this to be able to provide the energy required by the flagellar dynein ATPase to effect cellular motility.\textsuperscript{321 - 323} When the activity of spermatozoal CPT is specifically inhibited with 2,4-dinitrofluorobenzene, cell motility is disrupted and impeded as the cells lose movement in the distal regions of their flagellae. The motility of permeabilised cells can be restored with either ATP or a mixture of ADP and PCr, although the latter combination is ineffective in the presence of 2,4-dinitrofluorobenzene. These observations have led to a proposal that PCr carries high-energy phosphoryl groups from the mitochondria to ATPase along the length of the flagellum.\textsuperscript{322 - 324}

The recent description of distinct CPT isoenzymes in the mitochondria (M\textsubscript{M}) and flagellae (BB) of chicken and human spermatozoa suggests that a similar shuttle may be at work in these cells also.\textsuperscript{325}

1.3.4 Testicular creatine metabolism.

The mammalian testis is a major site of creatine localisation when compared with other organs of the body (Table 1.4). The presence of CPT activity within the testis of the rat allows for the utilisation of creatine by way of the transphosphorylation reaction with ATP,\textsuperscript{285, 326} although relatively little testicular creatine is found as PCr (0-7%, cf. 40-70% for muscle).\textsuperscript{248 - 250, 252, 266, 328 - 330}
Table 1.4. Comparison of the creatine content of the testis with that of other organs. Evaluation of creatine content of various organs from mature animals.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Creatine Content (μmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Rat</td>
</tr>
<tr>
<td>Reference</td>
<td>246</td>
</tr>
<tr>
<td>Testis</td>
<td>22.8</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>34.7</td>
</tr>
<tr>
<td>Cardiac Muscle</td>
<td>16.6</td>
</tr>
<tr>
<td>Brain</td>
<td>11.8</td>
</tr>
<tr>
<td>Liver</td>
<td>3.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.2</td>
</tr>
<tr>
<td>Species</td>
<td>Rat</td>
</tr>
<tr>
<td>Reference</td>
<td>247</td>
</tr>
<tr>
<td>Testis</td>
<td>20.5</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>38.5</td>
</tr>
<tr>
<td>Cardiac Muscle</td>
<td>15.0</td>
</tr>
<tr>
<td>Brain</td>
<td>11.1</td>
</tr>
<tr>
<td>Liver</td>
<td>2.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>ND</td>
</tr>
<tr>
<td>Species</td>
<td>Rat</td>
</tr>
<tr>
<td>Reference</td>
<td>248</td>
</tr>
<tr>
<td>Testis</td>
<td>22.7</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>38.0</td>
</tr>
<tr>
<td>Cardiac Muscle</td>
<td>14.7</td>
</tr>
<tr>
<td>Brain</td>
<td>ND</td>
</tr>
<tr>
<td>Liver</td>
<td>2.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>ND</td>
</tr>
<tr>
<td>Species</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Reference</td>
<td>327</td>
</tr>
<tr>
<td>Testis</td>
<td>6.5</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
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</tr>
<tr>
<td>Cardiac Muscle</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>2.5</td>
</tr>
<tr>
<td>Liver</td>
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</tr>
<tr>
<td>Kidney</td>
<td>0.9</td>
</tr>
<tr>
<td>Species</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Reference</td>
<td>327</td>
</tr>
</tbody>
</table>

*Other reported values for testicular creatine content (quoted here as μmol/g tissue) in mammals, measured without comparison to that of other organs: rat, 17.2-23.0; mouse, 21.0; rabbit, 9.4; cat, 5.0; guinea pig, 2.5-2.6.251, 252

^Not determined

^Reported as ‘negligible’

Generally, sites of creatine synthesis and utilisation in the rat are segregated,253 but the testis is unique in that it synthesises creatine at a considerable rate (approximately 0.2 μmol/organ/30 min which represents 1% of the organ content, estimated from the data of Koszalka).251, 331 Therefore the testis represents an unusual situation as regards creatine metabolism, having a significant creatine pool, and the capacity to both synthesise and utilise creatine.246 - 252, 266, 285, 326, 331 Although creatine has been postulated to play a role in sperm motility in a number of species,273, 321 - 325 any further role in the testis as a whole is as yet unclear.
1.4 BACKGROUND AND AIMS OF THE PROJECT.

1.4.1 Starting point for the project.

During investigations into the biochemical changes induced by the administration of various nephrotoxicants, Drs. Timbrell and Nicholson of this laboratory used proton nuclear magnetic resonance spectroscopy (NMR) to examine the metabolic profiles of urine after the exposure of rats to cadmium.

Proton NMR is a broad-substrate analytical technique that allows for the simultaneous measurement of a wide range of different compounds. After the exposure of rats to cadmium, an alteration in the excretion of a number of urinary metabolites was noted, including a dose-related elevation in the excretion of creatine. Doses of cadmium that induced creatinuria were also shown to cause testicular necrosis. It was concluded that the elevation in creatine excretion was a direct result of this damage, since it did not occur after the exposure of either female or orchidectomised male rats to cadmium.

In a later study, administration of the germ cell toxicant 2-ME to male rats caused an elevation in urinary creatine excretion, but no such effect was observed when female rats were similarly exposed.

1.4.2 Aims of the project.

This project started with an attempt to determine the distribution of creatine within the testis, and to assess creatinuria as a non-invasive marker for testicular damage, by monitoring urinary creatine excretion after the administration of different cell-specific testicular toxicants. Subsequently, the metabolism of creatine within the testis was to be examined, with a view to determining the role of creatine within the testis.
CHAPTER 2

MATERIALS AND METHODS
2.1 MATERIALS.

2.1.1 Animals.

Male Hsd/Ola : Sprague Dawley rats were purchased from Harlan Olac (Bicester) at least one week prior to use. They were housed on sawdust, and allowed free access to food (Quest Rat and Mouse Maintenance Diet, Lillico, Betchworth, Surrey) and tap water.

2.1.2 Chemicals.

L-[guanidino-^14C]Arginine monohydrochloride (54.4 μCi/μmol) and [^{13}C]glycine (56 μCi/μmol) were obtained from Amersham International plc (Aylesbury, Bucks), and [^{1-14}C]creatine hydrate (13.5 μCi/μmol) from ICN Radiochemicals (Irvine, CA, U.S.A.).

DPP was supplied by the Eastman Kodak Co. (Liverpool). [^{1-14}C]GAA, EDS and MEHP were synthesised as described below (§2.2). All other chemicals were of the highest quality commercially available and were supplied by Sigma, Aldrich or British Drug Houses (all of Poole, Dorset, U.K.) or Boehringer Corporation London.

Liquid scintillation counting was carried out using ‘Aquasol’ obtained from New England Nuclear plc (Edinburgh, Scotland).

Deionised water was prepared from distilled water using an Elgastat UHQ deioniser (Elga UK, High Wycombe, Bucks.).

2.1.3 Culture stocks.

Collagenase and DNase-I were purchased from Sigma. Phosphate buffered saline (PBS, Dulbecco ‘A’) tablets were from Oxoid Ltd. (Basingstoke, Hants.).

All other cell culture stocks and reagents were supplied by Gibco Ltd. (Paisley, Scotland). Nylon mesh was from Henry Simon Precision Textiles Ltd. (Manchester), or from Lockertex (Warrington). All plastic ware was supplied by Steralin Ltd. (London).
2.2 CHEMICAL SYNTHESIS.

2.2.1 Synthesis of [\(^{14}\text{C}\)]GAA.

[\(^{14}\text{C}\)]GAA was synthesised from [\(^{14}\text{C}\)]glycine as described by Dubnoff.\(^{336}\)

Glycine (383 mg, 5.10 mmol) was dissolved in water (4 ml) with [\(^{14}\text{C}\)]glycine (9 \(\mu\)Ci). Cyanamide (500 mg) and concentrated ammonia solution (3 drops) were added. The solution was left at room temperature for 72 h, and then at 4°C overnight. The precipitated solid was washed five times by repeated recrystallisation from the minimum of hot water, and then dried overnight at 70°C. The final yield of [\(^{14}\text{C}\)]GAA was 25.5% (152.5 mg, 1.30 mmol), specific activity 1.72 \(\mu\)Ci/mmol. The chemical purity was >99%, as indicated by elemental analysis (calculated for C\(_3\)H\(_7\)N\(_3\)O\(_2\): C, 30.77; H, 6.46; N, 35.87; found - C, 36.67; H, 6.16; N, 31.79), and high-performance liquid chromatography (HPLC, \S2.10). Radiochemical purity, also determined by HPLC, was found to be >95%.

2.2.2 Synthesis of EDS.

EDS was synthesised by the method of Jackson and Jackson.\(^{337}\)

Dry ethylene glycol (9.40 g, 151 mmol) and pyridine (45 ml) were cooled in an ice-salt bath and stirred. The temperature was maintained below 10°C as methane sulphonylchloride (23 g) was added drop-wise. The ice bath was then removed, and when the reaction mixture had reached room temperature it was added, with continual stirring, to a prepared ice-acid mixture (45 ml concentrated sulphuric acid added to 0.75 l crushed ice). The mixture was stirred until the separating oil had solidified.

The solid was isolated by filtration under suction, washed four times with cold water and once with cold methanol. It was recrystallised five times from chloroform-methanol (1:1 v/v). The final yield of EDS was 34.6% (11.45 g, 52 mmol) and it was >99% pure, as indicated by elemental analysis (calculated for C\(_4\)H\(_{10}\)S\(_2\)O\(_6\): C, 22.01; H, 4.62; S, 29.38; found - C, 22.16; H, 4.69; S, 29.17) and 60 MHz proton NMR. The product had a melting point of 43.5-46.5°C (the melting point of EDS is 45°C), and its identity was confirmed by
electron impact and fast atom bombardment mass spectrometric analyses.

2.2.3 Synthesis of MEHP.

MEHP was synthesised by Dr. R. Purchase of BIBRA according to the method of Albro et al.\textsuperscript{71}

Briefly, phthalic anhydride (148 mg) was refluxed with 2-ethyl-1-hexanol (0.65 ml) in toluene (25 ml) for 7 h. The monoester was extracted into 0.4 M potassium carbonate and, after acidification with hydrochloric acid, was extracted back into diethyl ether. After evaporation of the solvent the resultant MEHP had a purity of >99% (as determined by HPLC) and its identity was confirmed by elemental analysis and NMR spectrometry.
2.3 THE DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) METHOD FOR THE DETERMINATION OF CREATINE IN URINE.

2.3.1 Introduction.

During investigations in this laboratory into urinary creatine excretion, the measurement of creatine concentration in urine samples has been accomplished by the use of an enzymatic kit designed for the determination of creatinine. The kit comprises two reagents (R₁ and R₂) which allow for the measurement of creatinine through its hydrolysis to creatine by the enzyme creatininase,

\[
\begin{align*}
\text{creatine} & \xrightarrow{\text{creatininase}} \text{creatinase} \\
\text{creatine} & \xrightarrow{\text{creatininase}} \text{creatinase} \\
\text{sarcosine} & \xrightarrow{\text{sarcosine oxidase}} \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 & \xrightarrow{\text{peroxidase}} \text{dye formation}
\end{align*}
\]

Creatine is measured as a background contaminant by R₁. Unfortunately, since R₁ includes both creatinase and sarcosine oxidase, this method cannot distinguish between creatine and sarcosine in samples. Other methods are required to confirm high creatine measurements.

In the previous studies, confirmation was achieved by proton NMR spectroscopy. However, this method is time consuming, expensive, and not readily amenable to routine analysis, since it requires the use of heavily subscribed equipment.

In order to circumvent these problems, a chromatographic method for creatine analysis, as a back-up for the routine use of the enzymatic assay kit, has been developed.
2.3.2 Theoretical considerations for the chromatography of creatine.

The components of aqueous solutions (e.g. urine) are separated by reverse-phase chromatography, in which the mobile phase is more polar than the stationary phase. In this type of system, the stationary phase is a hydrophobic group (e.g. C<sub>18</sub>) attached to a silica support, with the mobile phase based upon water or some other polar solvent. Components are then separated by their relative abilities to diffuse into the stationary phase. Those components whose ionic characteristics cannot be suppressed sufficiently by altering the pH of the mobile phase can be retained on the column by ion-pairing with a detergent.\(^{338,339}\)

Creatine forms a zwitterion in solution, with a highly basic guanidino group (\(pK_a = 14.3\)) and a carboxylic group (\(pK_a = 2.63\)).\(^{245}\) The ionic character of the guanidino group cannot be suppressed within the operational range for silica-based columns (pH 2.0-7.5), but a low pH will suppress the carboxylate ion and will maximally ionise the basic group, leaving the compound in a suitable condition for ion-pairing with an alkylsulphonate counterion.

2.3.3 Method development.

The HPLC system used comprised an Altex 421 controller and model 110 A pump, a guard column (25 × 4 mm i.d.) packed with Vydac reverse phase material (Varian), a main column (300 × 4 mm i.d.) packed with Micropak MCH-10 ODS (Varian), a Cecil model CE 2112 reference channel variable wavelength UV monitor and an Altex C-RIA chart recorder. The sensitivity of the spectrophotometer was adjusted so that 0.100 absorbance units gave full-scale deflection of the chart recorder.

Initially the method was developed using aqueous solutions of creatine and creatinine (considered to be a possible close-eluting contaminant in any chromatogram). Once the conditions for retention and separation of creatine and creatinine had been defined, then the mobile phase was adjusted in order to obtain the optimum separation of creatine from other components in genuine samples of rat urine.
Creatine and creatinine were dissolved in deionised water to give final concentrations of 0.1 and 1.0 mg/ml respectively (approximating to those found in normal rat urine). These standards were diluted 1:40 with the mobile phase buffer.

The choice of counterion was determined by measuring the retention times and separation of the creatine and creatinine peaks with alkylsulphonates and alkylsulphates of different carbon chain length. For this purpose, the pH of the mobile phase was set at 2.0, to give maximum suppression of the carboxylate group of creatine. Methane sulphonate and ethane sulphonate gave low retention and poor separation, whereas octane sulphonate and dodecyl sulphate gave long retention times and broad peaks. Better retention, separation and peak shape were obtained with butane sulphonate, but optimum chromatography was obtained with hexane sulphonate, and so this counterion was selected for use.

When urine samples (20 μl of a 1:40 dilution with the mobile phase) were applied to the column, it was found that creatine was poorly separated from unidentified interfering components that eluted soon after. Consequently, the pH of the mobile phase was altered, and at pH 2.25 complete separation was effected (Figure 2.1).

2.3.4 Method validation.

The method was validated by determining the linearity of response, the reproducibility of measurements, and by comparing the results obtained from selected samples with those obtained with a previously established enzymatic method.

i) 'Spiking' of urine samples. Samples of urine taken from each of two normal, untreated, 4-week old rats were 'spiked' with creatine (0.538 or 1.075 mg/ml). The samples were diluted 1:40 with the mobile phase and an aliquot (20 μl) applied to the column. The concentration of creatine was measured in the samples, converted to the pre-dilution values, and then correlated with the known additions made (Table 2.1).
Figure 2.1. The separation of creatine from other urinary metabolites by HPLC. HPLC profiles of urine from a normal rat (A) with no additions (measured [creatine] = 0.014 mg/ml), and (B) 'spiked' with 0.538 mg/ml creatine (measured [creatine] = 0.530 mg/ml). The creatine peak is shown with an arrow.

ii) Dilution of samples. Aliquots (20 µl) of the 'spiked' samples described above were injected onto the column following 1:20, 1:40 and 1:80 dilutions with the mobile phase. The measured concentrations were converted to the pre-dilution values and correlated with the known additions (Table 2.1).

iii) Correlation of the HPLC method with an enzymatic method. Samples of urine from ten animals were analysed by both the HPLC method and a commercial enzymatic kit purchased from Boehringer Corporation London. The enzymatic method is described more fully below (§2.6.1). The correlation between the two methods is shown in Figure 2.2, the regression line passes through the origin and has a slope of 0.968.
Table 2.1. Comparison of known and measured creatine additions to samples of normal rat urine. Samples of urine from two normal animals were spiked with creatine and diluted 1:20, 1:40, or 1:80 prior to analysis. The measured creatine concentrations were then correlated with the known additions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution factor</th>
<th>Creatine added (mg/ml)</th>
<th>Creatine found (mg/ml)</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:20</td>
<td>0.000</td>
<td>0.014</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>0.538</td>
<td>0.499</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:80</td>
<td>1.075</td>
<td>1.030</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1:20</td>
<td>0.000</td>
<td>0.101</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>1.075</td>
<td>1.131</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.2. Comparison of the HPLC and enzymatic methods for the measurement of urinary creatine.

From the results of the evaluation experiments it was concluded that the new HPLC method gave a linear response over the range of creatine concentrations of interest, and would also allow samples with very high concentrations of creatine to be diluted to within the working range. The method also correlated well with the enzymatic method that it was
developed to supplement and, since sarcosine was not retained by the column (not shown),
could therefore be used to confirm that measurements of high urinary creatine concentrations
made using the enzymatic kits were not a result of interference by sarcosine.

For many chromatographic techniques it is usual to add an internal standard (an
exogenous compound that has similar chromatographic properties, and that can be detected
with the same methods as the compound(s) of interest) to samples and standards in order to
minimise the errors arising as a result of liquid transfer, derivitisation, etc. Peaks of interest
in the sample and standard chromatograms are then expressed in terms of the internal
standard peak, and can thus be directly related to each other. However, urine is a highly
complex matrix, with many components that absorb light at a wavelength of 206 nm. The
complicated nature of the chromatograms obtained from monitoring the column eluent at
such a non-specific wavelength (Figure 2.1), meant that it was not possible to add an internal
standard that eluted close enough to creatine for optimum peak shape to be obtained.
However, although no internal standard was used, errors were minimised by the limited
sample preparation involved in the assay (i.e. a single dilution).

2.3.5 Procedure for the determination of creatine in urine by HPLC.
a) Mobile phase: 100 mM potassium chloride and 10 mM sodium 1-hexane
    sulphonate in water, pH 2.25. Dissolve 7.46 g potassium chloride
    and 1.882 g sodium 1-hexane sulphonate in 1 l of deionised water
    and adjust the pH to 2.25 with the minimum of hydrochloric acid.
b) Stationary phase: C_{18}, 10 μm, pellicular.
c) Flow rate: 1 ml/min for 8.5 min, increasing to 1.5 ml/min over 0.5 min and
    reverting to 1 ml/min after a further 2 min.
d) Temperature: ambient.
e) Detection: absorbance at 206 nm.
f) Sample preparation: dilute 1:40 with the mobile phase. Apply 20 μl to column.
2.4 PROCEDURES FOR THE STUDY OF TESTICULAR TOXICITY IN VIVO.

2.4.1 Treatment of animals.

Animals were housed individually in plastic metabolism cages under laboratory conditions (24 ± 2°C, 12 h light/dark cycle) for at least three days prior to the start of the experiment in order to allow them to adjust to their new environment.

2.4.2 Urine collection and storage.

Urine was collected over ice in 24 h periods, the volume noted, and then stored at -20°C prior to analysis, which was normally carried out within 24 h. After each collection the urine collection ducts of the cages were flushed clean of absorbent food debris and faecal matter with distilled water using a wash bottle with a directional nozzle.

The first urine collection was made in the 24 h period prior to dosing (-24-0h) in order to determine the normal creatine and creatinine excretion and urine output of the animals. The animals were dosed at t = 0 h and urine was collected in two periods over the next 48 h (i.e. 0-24 h and 24-48 h).

2.4.3 Experiment termination.

At the end of the experiment the animals were weighed and sacrificed by cervical dislocation. Larger animals were anaesthetised under diethyl ether prior to sacrifice. The testes were removed, weighed and placed in Bouin’s fixative for at least 24 h prior to processing into paraffin wax. Sections were cut and stained with haematoxylin and eosin (H&E) for examination by light microscopy.
2.5 PROCEDURES FOR TISSUE AND CELL CULTURE.

2.5.1 Preparation of isolated seminiferous tubules.

Seminiferous tubules were isolated from the testes of 4 or 16-17 week old rats essentially as described by Jutte et al.\textsuperscript{143}

*Materials.* Hanks' balanced salt solution (HBSS);\textsuperscript{340} collagenase solution (2 mg/ml, in HBSS). The final incubation medium consisted of HBSS supplemented with 2 mM glycine and 2 mM L-methionine.

*Method.* For each preparation a single animal was killed by cervical dislocation, older animals were first anaethetised with diethyl ether. The testes were removed, decapsulated and incubated with 10 ml collagenase solution at 37 °C in a shaking water bath (2 Hz). After 15 min HBSS (10 ml) was added, the tubules were allowed to settle and the supernatant was removed. The tubules were washed three times with HBSS and suspended in 20 ml incubation medium. This suspension was aliquoted out into 25 ml conical flasks and incubated in a water bath at 32 °C under an atmosphere of 5% CO$_2$/95% O$_2$.

2.5.2 Isolation of an interstitial cell population.

An unpurified interstitial cell population was obtained during the isolation of seminiferous tubules (§2.5.1). After the collagenase incubation, the tubules were allowed to settle and the supernatant was removed, centrifuged (45 g, 5 min), and washed three times with HBSS. The cells were finally resuspended in 10ml of incubation medium (§2.5.1) and this suspension was then aliquoted out into 10 ml conical flasks. The tissue was incubated under the same conditions as described above (§2.5.1).
2.5.3 Preparation of Sertoli-germ cell co-cultures (SGCC).

SGCC were prepared from the testes of 27-29 day old rats by the method of Gray and Beamand.53

Materials. HBSS without calcium and magnesium (Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS); trypsin/DNase-1 solution (0.125% trypsin, 10 \(\mu\)g/ml DNase-1 in Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS); collagenase solution (0.1% in Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS). The culture medium consisted of Eagle’s minimal essential medium (EMEM), supplementation with 4 mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin and 10% foetal calf serum.

Method. Testes from 3-6 animals were decapsulated, gently teased and then chopped with a scalpel into pieces approximately 3-4 mm\(^2\). The tissue was washed once with Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS and then incubated with trypsin/DNase-1 solution (10 ml/g tissue) for 30 min at 37 °C. The flask was shaken intermittently in order to facilitate the dispersion of the tissue into individual tubules. The trypsin solution was decanted through a 100 \(\mu\)m nylon mesh and the retained tubules were washed with a stream of 100 ml Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS. The tubules were resuspended in collagenase solution (10 ml/g tissue) and were incubated at 37 °C with frequent shaking for approximately 15 min, until the tissue was reduced to 1-2 mm fragments. The suspension was filtered through a 75 \(\mu\)m nylon mesh and the retained tissue was washed with 100 ml Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS. Tissue was recovered from the mesh by backwashing with 100-200 ml of culture medium, glutinous tissue removed, and the remainder was dispersed into a suspension of single cells and small tubular fragments by gentle pipetting. The cell suspension was plated out into 6 × 35 mm multiwell plates or 50 mm petri dishes. Cultures were incubated at 32 °C under a humidified atmosphere of 5% CO\(_2\)/95% air.

After 24 h incubation the medium was replaced with serum-free medium, which was replaced every 24 h for the remainder of the experiment.

Forty eight hours after preparation, the cultures consisted of a confluent layer of Sertoli cells with germ cells distributed on top (Figure 2.3).
Figure 2.3. Sertoli-germ cell co-cultures. Cultures prepared on glass coverslips as described (§2.5.3) were fixed with Bouin's fixative for 10 minutes prior to staining with H&E. The cultures consist of a monolayer of Sertoli cells attached to the plastic substrate (lying below the plane of focus of these photographs) to which are attached different sub-populations of germ cells. Magnification (A) ×160, (B) ×640.
2.5.4 Preparation of Sertoli cell-enriched cultures (SCEC).

SCEC were prepared by selectively removing the germ cells from the co-cultures described previously (§2.5.3) with a hypotonic shock treatment. This treatment leaves a Sertoli cell culture which is almost completely devoid of germ cells (Figure 2.4), whilst functional characteristics of the Sertoli cells are retained.

Materials. Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) in water (20 mM, pH 7.4).

Method. After the cultures had been incubated for 48 h they were exposed to Tris-HCl for 5 min, and the dishes were gently agitated to facilitate germ cell detachment. The hypotonic solution was then gently aspirated off and replaced with fresh medium. Cultures were incubated for a further 24 h before use.

Cell viability was assessed by vital dye exclusion (§2.5.6). The only non-viable cells were those few contaminating germ cells that hadn't detached from the culture during the hypotonic shock treatment. The viability of the Sertoli cells was estimated to be >99%.
Figure 2.4. Sertoli cell enriched cultures. Cultures prepared on glass coverslips and the germ cells were removed 48 h later as described (§2.5.3 & §2.5.4). SCEC were fixed with Bouin's fixative for 10 minutes prior to staining with H&E. The cultures consist of a monolayer of Sertoli cells attached to the plastic substrate with no germ cells lying on top. Magnification (A) $\times 160$, (B) $\times 640$. 
2.5.5 Preparation of isolated mixed germ cell cultures.

Germ cells were isolated from the testes of 30-31 day old rats, essentially as described by Meistrich. Materials. Phosphate-buffered saline, supplemented with 1 mg/ml D-glucose (PBSG); collagenase solution 0.1% in PBSG; trypsin/DNase-I solution (0.25% trypsin, 1 μg/ml DNase-I in PBSG); 1% bovine serum albumin (BSA) in PBSG; BSA/DNase-I solution (0.2% BSA, 2 μg/ml DNase-I in PBSG); BSA solution (2 mg/ml in deionised water). The final culture medium consisted of EMEM supplemented with 2 mM L-glutamine, 6 mM L-lactate, 2 mM pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin.

Method. The testes from one 30-31 day old rat were removed, carefully decapsulated and incubated for approximately 17 min in collagenase solution (10 ml) at 32 °C in a shaking water bath (2.3 Hz) until the tissue had dispersed into separated tubules. The tubules were allowed to settle and the solution was aspirated off, after which they were washed twice with 10 ml PBSG and then incubated in 10 ml trypsin/DNase-I solution at 32 °C until the tissue had broken down into small fragments and single cells. Undigested glutinous tissue was removed, and the contents of the flask were gently pipetted to suspend the cells and disperse clumps. The suspension was layered over 10 ml of 1% BSA in a 50 ml round bottomed centrifuge tube and centrifuged at 45 g for 5 min. The solution was aspirated off and the pellet resuspended in 15 ml BSA/DNase-I solution. The cell suspension was filtered through a 75 μm nylon mesh, the filtrate was centrifuged (45 g, 5 min) and the pellet resuspended in culture medium to give a final cell density of 2 × 10^6/ml. Aliquots (10 ml) of this cell suspension were cultured in 100 mm petri dishes, which had been preincubated overnight at room temperature with 2 mg/ml BSA, at 32 °C under a humidified atmosphere of 5% CO₂/95% air.

The cellular composition of the isolated germ cell population was determined in the following manner. A small sample (0.1 ml) of the cell suspension was pelleted, and the cells resuspended in 4% formaldehyde. This suspension was smeared onto a microscope slide.
and allowed to dry prior to Giemsa staining and identification.

2.5.6 Assessment of cell viability by trypan blue exclusion.

Cell viability was routinely assessed, either qualitatively or quantitatively, by the ability to exclude the vital dye trypan blue.

Principle. This method takes the integrity of the plasma membrane as the parameter to distinguish viable cells and assumes that leakage of this membrane is an irreversible stage of cell death. When cells are added to a solution containing a vital dye, which cannot normally cross the plasma membrane, non-viable cells can be identified as those which are stained. Viable cells appear pale or colourless.

Reagents. Isotonic saline (0.9% NaCl in water); trypan blue solution (0.4% in saline).

Method. Sertoli cell viability was qualitatively assessed in the following manner. The medium was removed from randomly selected dishes and replaced with enough trypan blue solution to cover the cells. After 1-2 min the solution was removed, the cultures were washed twice with saline and then examined by the naked eye and under light microscopy (magnification ×100). The percentage viability was estimated as the area occupied by viable cells compared to the total area under examination.

The viability of germ cells was determined as follows. An aliquot (0.1 ml) of the cell suspension was added to trypan blue solution (0.1-0.9 ml) in a small test-tube. After 1-2 min the cells were counted in an improved Neubauer haemocytometer and the viability expressed as the percentage of cells able to exclude the dye.

The disadvantage of this method is that it cannot be used to accurately determine quantitatively the viability of cells which are adhered to a culture substrate (e.g. growing as a monolayer), since to obtain such cells as a suspension (necessary for counting on a haemacytometer) requires their removal from their support by trypsinisation. This may destroy non-viable intact cells in preference to viable ones, thus giving a false high viability.
2.6 DETERMINATION OF URINARY METABOLITES.

2.6.1 Creatine and creatinine (enzymatic method).

Urinary creatine and creatinine levels were routinely assayed by the method of Siedel et al.,\textsuperscript{346} using a commercial kit from Boehringer Corporation London.

*Principle.* This assay is based upon the hydrolysis of creatinine by the enzyme creatininase (creatinine amidohydrolase) to produce creatine which is in turn hydrolysed by creatinase (creatinine amidinohydrolase) to sarcosine. Sarcosine oxidase (sarcosine: oxygen oxidoreductase (demethylating)) oxidises sarcosine to produce hydrogen peroxide, which with a phenol derivative and 4-aminophenazone, in a reaction catalysed by peroxidase (donor: hydrogen-peroxide oxidoreductase), produces a red benzoquinone-imine dye:

\[
\text{creatinase} \quad \text{creatinine} + \text{H}_2\text{O} \rightarrow \text{creatine} \\
\text{creatinase} \quad \text{creatine} + \text{H}_2\text{O} \rightarrow \text{sarcosine} + \text{urea} \\
\text{sarcosine oxidase} \quad \text{sarcosine} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{glycine} + \text{HCHO} + \text{H}_2\text{O}_2 \\
\text{peroxidase} \quad \text{H}_2\text{O}_2 + \text{phenol derivative} + 4\text{-aminophenazone} \rightarrow \text{red benzoquinone-imine dye}
\]

*Reagents.* Reagents I and II (R\textsubscript{1} and R\textsubscript{II}); creatine standard (17.6 µg/ml, in deionised water); creatinine standard (20 µg/ml, supplied with the kit). R\textsubscript{II} was prepared from R\textsubscript{1} by the addition of creatininase. The composition of R\textsubscript{II} was as follows: 0.15 M potassium phosphate (pH 7.9), 10 µM potassium hexacyanoferrate, 8.6 mM 2,4,6-tribromo-3-hydroxybenzoic acid, 0.8 mM 4-aminophenazone, 5 mM sodium cholate, 0.25% detergent, >20 U/ml creatininase, >10 U/ml creatinase, >5 U/ml sarcosine oxidase, >1 U/ml peroxidase, >10 U/ml ascorbate oxidase, >1 U/ml lipase.

*Method.* Urine samples were diluted 1:20 with deionised water. Duplicate aliquots (50 µl) were mixed with 1 ml of either R\textsubscript{1} or R\textsubscript{II} and incubated at room temperature for 20 min. Parallel incubations were carried out with creatine and creatinine standards, and reagent blanks (R\textsubscript{B}, R\textsubscript{II}B - using deionised water instead of sample). The absorbance (A) at 510 nm...
was measured using a Shimadzu MPS-2000 spectrophotometer and the concentrations of creatine ([Cr]) and creatinine ([Cm]) calculated as follows:

\[
[Cr] = \frac{AR_{sample} - AR_B}{AR_{standard} - AR_B} \times 17.6, \mu g/ml
\]

\[
[Cm] = \frac{AR_{sample} - AR_{sample} - AR_B + AR_B}{AR_{standard} - AR_{standard} - AR_B + AR_B} \times 20.0, \mu g/ml
\]

The 24 h excretion calculated by multiplying these values by the 24 h urine volume.

### 2.6.2 Creatine (HPLC method).

In order to confirm elevated creatine concentrations in urine as measured by the enzymatic method (§2.6.1), a HPLC system was developed in this laboratory to separate out urinary constituents (§2.3).

**Principle.** The principle behind this technique has been described previously (§2.3.2 & §2.3.3).

**Reagents.** The composition of the mobile phase is described in §2.3.5a; creatine standards (0-1 mg/ml in distilled water).

**Method.** Urine samples and standards were diluted with the mobile phase (1:40) and duplicate 20 μl aliquots were applied directly to the column. Peak heights on the printout charts were measured by hand and concentrations in the samples calculated by direct reference to the standard curve.
2.7 DETERMINATION OF INTRACELLULAR METABOLITES.

2.7.1 Creatine.

Tissue creatine levels were determined by the method of Yasuhara et al.\textsuperscript{307}

**Principle.** The method is similar to that of Siedel et al.,\textsuperscript{346} except that it is the production of formaldehyde from the oxidation of sarcosine that is monitored. Formaldehyde is oxidised by formaldehyde dehydrogenase (formaldehyde: NAD\textsuperscript{+} oxidoreductase) to formate and the concomitant increase in absorbance at 340 nm as NAD\textsuperscript{*} is reduced to NADH is measured:

\[
\text{HCHO} + \text{NAD}\textsuperscript{*} \rightarrow \text{HCOOH} + \text{NADH} + \text{H}^+ \]

Since the components of this assay can be purchased individually, then a ‘sarcosine oxidase-formaldehyde dehydrogenase blank’ can be prepared to eliminate interference by endogenous sarcosine and formaldehyde.

**Reagents.** Reagent I (R\textsubscript{i}) consisted of all the components of the assay system except creatinase; reagent II (R\textsubscript{ii}) was prepared from R\textsubscript{i} by the addition of creatinase (the final composition of R\textsubscript{ii} was as follows: 0.1 M potassium phosphate (pH 7.8), 0.1 mM NAD\textsuperscript{*}, 10 U/ml creatinase, 1.5 U/ml sarcosine oxidase, 0.15 U/ml formaldehyde dehydrogenase); creatine standard (44 \textmu g/ml in water).

**Method.** Duplicate aliquots (0.1 ml) of samples were mixed with 1 ml of either R\textsubscript{i} or R\textsubscript{ii} and left to incubate at 37 °C for 15 min. The absorbance (A) at 340 nm was then measured with a Shimadzu MPS-2000 spectrophotometer. Reagent blanks (R\textsubscript{i}B, R\textsubscript{ii}B) were performed by using 0.1 ml water instead of sample. The concentration of creatine ([Cr]) was calculated by reference to the standard solution using the formula below,
2.7.2 ATP.

ATP was measured by a bioluminescence method.\textsuperscript{347}

*Principle.* A crude extract of luciferase (*Photinus* luciferin: oxygen 4-oxidoreductase (decarboxylating, ATP-hydrolysing)) from firefly tails hydrolyses ATP in the presence of luciferin and oxygen to emit light:

\[
\text{luciferase (E) + luciferin (LH}_2 \text{ + ATP} \rightarrow E.LH_2\text{AMP + PP}_i \\
E.LH_2\text{AMP + O}_2 \rightarrow E + \text{CO}_2 + \text{AMP + oxyluciferin + light}
\]

Since one photon of light is produced for each molecule of ATP consumed, then a photon detection system can be calibrated to estimate cellular ATP levels.

*Reagents.* 26.7 mM Magnesium sulphate/3.3 mM potassium dihydrogen phosphate/33.3 mM sodium arsenate (pH 7.4); reconstituted firefly extract; ATP standards (0-40 μM in 10% trichloroacetic acid (TCA)).

*Method.* Buffer (2 ml) was pipetted into a 12 × 75 mm plastic test-tube along with 10 μl of sample. Firefly extract (0.1 ml) was added, the solution vortex mixed, and 15 s after the addition of the extract the luminescence of the sample was measured using a Thorn EMI photon detection unit (Mk II control unit, Fact 50 Mk III cooling unit and T1500 photon detector unit) coupled to an Amstrad PC1512 microcomputer. The ATP content of the samples was determined by reference to the standard curve.

2.7.3 PCr.

PCr was determined by a bioluminescence method adapted from that for ATP.\textsuperscript{348}

*Principle.* CPT transfers the high energy phosphoryl group from PCr to ADP.
The amount of ATP produced is stoichiometrically related to the PCr content of the sample, and can be measured as described (§2.7.2).

Reagents. 26.7 mM Magnesium sulphate / 3.3 mM potassium dihydrogen phosphate / 33.3 mM sodium arsenate (pH 7.4); 20 mM AMP, 50 μM ADP; reconstituted firefly extract; CPT solution (4000 μU/ml); PCr standards (0-25 μM in saline).

Method. Buffer (1.9 ml), AMP / ADP solution (0.1 ml) and a 10 μl aliquot of the sample were placed in a 12 x 75 mm plastic test-tube. Firefly extract (100 μl) was added (time, t = 0 s) and the tube contents vortex mixed. At t = 90 s the background luminescence was measured. CPT (0.25 ml) was added at t = 120 s and mixed. The luminescence was measured again at t = 180 s. The light output due to the presence of PCr was given by the difference between that at 180 s and that at 90 s. The PCr content of the samples was determined by reference to a standard curve prepared in the same manner.

2.7.4 Cellular protein.

Protein was determined by the method of Lowry et al. The method relies upon the reduction of a phosphomolybdate-phosphotungstate reagent by a copper-protein complex under alkaline conditions. Tyrosine and tryptophan residues in the protein appear to reduce Cu²⁺ ions to Cu⁺, which in turn catalyse the reduction of the phosphomolybdate-phosphotungstate anions. The result is a blue chromophore with an absorption maximum at 750 nm.

Reagents. Alkaline copper tartrate (0.01% copper sulphate and 0.02% sodium potassium tartrate in 2% disodium sulphate / 0.4% sodium hydroxide); phenol reagent (commercial Folin & Ciocalteu’s reagent, diluted 1:1 with distilled water); BSA standards (0-500 μg/ml in water).

Method. Samples were solubilised, where necessary, by dissolving in 1M NaOH to
give a final protein concentration of approximately 200-400 μg/ml.

Duplicate aliquots (50 μl) were mixed with alkaline copper tartrate solution (2 ml) and allowed to stand at room temperature for 10 min. Phenol reagent (0.2 ml) was then added and the solution immediately mixed. After 30 min the absorbance was measured at 750 nm using a Pye Unicam SP6-550 UV/VIS spectrophotometer. The concentration of protein in the samples was determined by reference to the standard curve.

2.7.5 CPT.

CPT activity was determined by a standardised method.\textsuperscript{358} This is a rate assay, and was carried out on a Shimadzu MPS-2000 spectrophotometer coupled to a PR-3 graphic printer. The assay temperature was maintained with a Howe TQ60 thermocirculator.

Principle. CPT catalyses the transfer of phosphoryl groups from PCr to ADP, producing ATP and creatine. ATP is used by hexokinase (ATP: d-Hexose-6-phosphotransferase) to phosphorylate d-glucose. The resulting d-glucose-6-phosphate is oxidised by d-glucose-6-phosphate dehydrogenase (d-glucose-6-phosphate: NADP\(^+\) 1-oxidoreductase), and the rate of increase in absorbance as NADP\(^+\) is concomitantly reduced to NADPH is a measure of creatine kinase activity (assuming that all other enzyme activities are not rate-limiting),

\[
\begin{align*}
\text{PCr} + \text{ADP} & \xrightarrow{\text{CPT}} \text{ATP} + \text{creatine} \\
\text{ATP} + \text{d-glucose} & \xrightarrow{\text{hexokinase}} \text{d-glucose-6-phosphate} + \text{ADP} \\
\text{d-glucose-6-phosphate} + \text{NADP}^+ & \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosphoglucono-δ-lactone} + \text{NADPH} + \text{H}^+
\end{align*}
\]

Reagents. 0.1 M Imidazole acetate buffer (pH 6.5), 2 mM ADP, 20 mM d-glucose, 2 mM NADP\(^+\), 10 mM magnesium sulphate, 5 mM AMP, 10 μM P\(^1\),P\(^5\)-di(adenosine-5')pentaphosphate, 20 mM N-acetyl-L-cysteine, 2 mM ethylene diamine tetraacetic acid (EDTA),
3500 U/l hexokinase, 2000 U/l D-glucose-6-phosphate dehydrogenase; PCr (345 mM in water).

Method. Buffer solution (1 ml) and sample (0.1 ml) were mixed in a cuvette and allowed to equilibrate to the assay temperature (32 °C), about 5 min. PCr solution (0.1 ml) was added and thoroughly mixed. The steady-state reaction rate was monitored after the initial lag phase of 60 - 90 s. The increase in the rate of change of absorbance due to the presence of CPT was determined by subtraction of the rate prior to the addition of PCr from that following addition, after allowing for differences in reaction volumes. The enzyme activity was calculated as follows:

\[
\text{Catalytic concentration} = \frac{\frac{dA}{dt}}{E \cdot d \cdot \nu} = 1.905 \cdot \frac{dA}{dt}, \quad \text{U/ml}
\]

Where \( \frac{dA}{dt} \) is the rate of change in absorbance at 340 nm (min⁻¹), \( E \) is the molar extinction coefficient of NADPH at 340nm (i.e. 630 m²/mol), \( d \) is the path length (i.e. 0.01 m), and \( \nu \) is the volume fraction of sample (i.e. 0.1 ÷ 1.2 = 0.08333).

Unit definition. One unit is defined as the activity required to dephosphorylate one micromole of PCr per minute at 32 °C.
2.8 DETERMINATION OF METABOLITES SECRETED INTO THE CULTURE MEDIUM.

2.8.1 Creatine.

Creatine was assayed by a slight modification of the highly sensitive fluorometric method described by Fry and Morales.351

*Principle.* The assay is based upon the reaction between creatine and 1,2-carboxy phenylglyoxal,352 which is produced as the five-membered ring of ninhydrin is opened under attack by hydroxide ions:353

\[
\text{ninhydrin} + \text{OH}^- \rightarrow 1,2\text{-carboxy phenylglyoxal}
\]

\[
1,2\text{-carboxy phenylglyoxal} + \text{creatine} \rightarrow \text{fluorophore}
\]

*Reagents.* Zinc sulphate (0.15 M, in deionised water); barium hydroxide (saturated solution in deionised water); ninhydrin (1% solution in 95% ethanol); potassium hydroxide (10% solution in 95% ethanol); creatine standards (0-67.2 μM in the culture medium).

*Method.* Duplicate aliquots (0.2 ml) of medium were adjusted to 1 ml with deionised water, zinc sulphate solution (0.5 ml) and barium hydroxide (0.6 ml) were added sequentially, mixed thoroughly and the suspension centrifuged (2700 g, 5 min). An aliquot (1 ml) of the supernatant was removed and placed in a clean test tube. Ninhydrin (0.5 ml) and potassium hydroxide (0.5 ml) were added and immediately mixed. The tubes were then stored in the dark. Fluorescence was measured (\(\lambda_{ex} = 390 \text{ nm}, \lambda_{em} = 500 \text{ nm}\)) after 10 min using a Perkin-Elmer LS-3 fluorescence spectrometer. Sample fluorescence was adjusted to allow for interference (see below), after which the creatine content of the samples was determined by reference to the standard curve.

*Interference.* Although the reaction between creatine and ninhydrin under the conditions described is not the same as that between the α nitrogen of amino acids and ninhydrin, and therefore amino acids do not interfere in the assay, it is quite general for a number of guanidino compounds, and such compounds have the capacity to give
erroneously high measurements. Of the compounds that could participate in the fluorogenic reaction, only two (arginine and GAA) were considered likely to interfere, since these were the only guanidino compounds, other than creatine, observed in HPLC radiochromatograms from experiments in which Sertoli cells or seminiferous tubules were incubated in the presence of \(^{14}C\)-arginine (see Chapter 4), and arginine is also a major amino acid component of EMEM.

In order to circumvent this problem the fluorescence readings of samples were adjusted, to allow for GAA secretion and arginine utilisation, using the following formula (the derivation of which is given in the appendix on page 187):

\[
F' = 0.79F_s + F_0(0.19 + 0.002t)
\]

When the incubation medium was Earle's balanced salt solution (EBSS), which does not contain arginine, the formula was simplified:

\[
F' = 0.79F_s
\]

Where \(F'\) is the adjusted fluorescence (to be compared with the standard curve), \(F_s\) is the measured fluorescence of the sample, \(F_0\) is the background fluorescence of the medium (by linear regression from the intercept of the standard curve with the ordinate in Figure 2.5), and

![Figure 2.5. Standard curve for the fluorometric determination of creatine. This represents a typical standard curve prepared in EMEM. The regression lines for standard curves prepared in EBSS passed through the origin (i.e. \(F_0 = 0\)).](image-url)
t is the incubation time in hours.

FSH, dbcAMP, MEHP and cadmium and did not interfere with the assay at the highest concentrations at which they were used. However, 1,3-DNB was found to interfere with the assay at concentrations above 100 µM (data not shown).

2.8.2 L-Lactate.

L-Lactate was measured by a modification of the method described by Hohorst.\textsuperscript{356}

**Principle.** The method relies upon the interconversion between lactate and pyruvate catalysed by LDH,

\[
\text{L-lactate} + \text{NAD}^{+} \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^{+}
\]

Under normal conditions, the equilibrium lies far to the left and so it is forced to the right by removing the pyruvate as the hydrazone,

\[
\text{pyruvate} + \text{hydrazine} \rightarrow \text{pyruvate hydrazone}
\]

**Reagents.** Reagent I (R\textsubscript{i}) contained all of the components of the assay system except LDH; reagent II (R\textsubscript{ii}) was prepared from R\textsubscript{i} by the addition of LDH (the final composition of R\textsubscript{ii} was as follows: 0.5 M glycine / 0.4 M hydrazine sulphate (pH 9.0), 2 mM NAD\textsuperscript{+}, 10 U/ml LDH); 10% PCA; L-lactate standards (0-5 mM in culture medium).

**Method.** Aliquots of medium (50 µl or 100 µl) were deproteinised with PCA (25 µl) and 1 ml of R\textsubscript{i} and R\textsubscript{ii} were added to duplicate samples. After vortex mixing the samples were left to incubate at room temperature for 90 min. The absorbance at 340 nm was measured with a Shimadzu MPS-2000 spectrophotometer. The increase in absorbance (A) due to the presence of L-lactate was calculated ($\Delta A = AR_{ii} - AR_{i}$), and the concentration of L-lactate was determined by reference to the standard curve.
2.9  RADIOLABEL METABOLISM STUDIES.

2.9.1  The separation of \([^{14}\text{C}]\text{creatine}\) and \([^{14}\text{C}]\text{GAA}\) from radiolabelled precursors by HPLC.

Separation of \([^{14}\text{C}]\text{creatine}\) and \([^{14}\text{C}]\text{GAA}\) from \(L-[\text{guanidino}^{14}\text{C}]\text{arginine}\) or \([1^{14}\text{C}]\text{glycine}\) was effected by the following modifications to the HPLC method described in §2.3.5.

A) Halide salts cause corrosion in stainless steel systems,\textsuperscript{359} and so the potassium chloride / hydrochloric acid buffer was replaced with an acidified sodium sulphate solution. The final composition of the mobile phase was 10 mM sodium sulphate, 5 mM sulphuric acid and 5 mM sodium 1-hexane sulphonate (pH 2.2).

The new mobile phase was not a buffered system, the relatively low concentration of sodium sulphate (10 mM as opposed to 100 mM potassium chloride) merely serving to 'salt out' the arginine, which was otherwise strongly retained.

B) The flow program was altered to allow maximal separation of \([^{14}\text{C}]\text{creatine}\) and \([^{14}\text{C}]\text{GAA}\) from each other and from their precursors. The initial flow rate of 1 ml/min was maintained for 8 min when it was increased to 2 ml/min over a 1 min period. After 4 min it was further increased to 3 ml/min over a 1 min period. This flow rate was maintained for 16 min when it was returned to 1 ml/min over 3 min.

The method was validated with authentic samples of \(L-[\text{guanidino}^{14}\text{C}]\text{arginine}\), \([1^{14}\text{C}]\text{creatine}\), \([1^{14}\text{C}]\text{GAA}\) and \([1^{14}\text{C}]\text{glycine}\).

2.9.2  Sample preparation and analysis.

Where necessary, samples were mixed with a cold carrier solution, so that the radiolabelled compounds to be separated were in a concentration of at least 0.1 mg/ml. Protein was removed by boiling for 5 min, followed by centrifugation at 2700 \(g\) for 5 min. An aliquot (50 \(\mu\)l) of the solution was then applied directly to the column.

The eluent was collected into 'betavials' in 0.4 min fractions over the first 13 min.
using an LKB Bromma model 2112 Redirac fraction collector. 'Aquasol' (5 ml) was added to each vial and, after the contents had been thoroughly mixed, the radioactivity was measured with a LKB Wallac 1216 Rackbeta liquid scintillation counter.
2.10 RADIOLABEL UPTAKE STUDIES.

Unless indicated otherwise, the buffer solution used in these studies was 20 mM Tris (final pH 7.4) containing 5 mM potassium chloride, 0.9 mM calcium chloride, 0.5 mM magnesium chloride, 5.6 mM D-glucose, 6 mM L-lactate, 2 mM sodium pyruvate, and either 0.14 M sodium chloride or 0.14 M choline chloride (adapted from Daly and Seifter). Cells (0.5-1.0 x 10⁶) were incubated in a volume of 1 ml, either in a water bath at 32 °C or in a cold room at 4 °C. [7-¹³C]Creatine was added to a final activity of 0.125 μCi/ml.

At the end of the incubation, the cells were resuspended, removed to a 12 x 75 mm test-tube, centrifuged (45 g, 5 min) and the supernatant removed. The cells were washed three times with ice-cold saline containing 10 mM unlabelled creatine, and dissolved in sodium hydroxide solution (1 M, 1 ml).

An aliquot of the digest was placed in a betavial and hydrochloric acid (1 M, 0.5 ml) and 'Aquasol' (4 ml) added. A blank count was carried out using sodium hydroxide solution (0.5 ml, 1 M). This was subtracted from the radioactivity measured in the samples to obtain the activity due to [1-¹³C]creatine. The uptake of creatine by the cells was calculated as follows:

\[
\text{Creatine uptake} = \frac{dpm_s - dpm_b}{f \cdot v \cdot SA_{cr}}, \text{ pmol}
\]

Where \(dpm_s\) is the activity of the sample, \(dpm_b\) is the activity of the blank, \(f\) is the conversion factor for dpm and pCi (1 pCi = 2.22 dpm), \(v\) is the volume fraction counted (i.e. 0.5 ml), and \(SA_{cr}\) is the specific activity of the creatine in the incubation solution.

The remainder of the digest was used to determine the protein content of the sample (§2.7.4).
2.11 DATA ANALYSIS.

Statistical and regression analyses of data were carried out using a Casio fx-50F personal scientific calculator. Statistical analyses of data were carried out with Student's two tailed t-test. The level of significance was set at 5% (i.e. \( P < 0.05 \)).

Analysis of uptake kinetics was performed on a BBC microcomputer, using the 'Enzpack' statistical package.
CHAPTER 3

URINARY CREATINE PROFILES AFTER ADMINISTRATION OF
CELL-SPECIFIC TESTICULAR TOXICANTS TO THE RAT
ABSTRACT.

Cell-specific testicular toxicants have been used to examine the distribution of creatine within the rat testis. Groups of rats were administered various single doses of a germ cell toxicant (MAA), one of two Sertoli cell toxicants (DPP or 1,3-DNB), or a Leydig cell toxicant (EDS). Urinary creatine and creatinine levels were monitored in 24 h periods over the following 48 h, after which time the testes were removed, weighed and, after processing, sections were examined by light microscopy.

All four compounds caused reductions in testis weight and produced degenerative changes in their respective target cells, with DPP and 1,3-DNB also causing alterations within the germ cell population, secondary to their effects upon the Sertoli cells. In addition, MAA, DPP and 1,3-DNB all caused large, transient elevations in urinary creatine excretion and the creatine:creatinine ratio (Cr:Crn) within 24 h, which returned to normal levels within 48 h after dosing. EDS had no such effect.

It is concluded that creatine is associated with the cells of the seminiferous epithelium, and that elevated urinary excretion of creatine may serve as a non-invasive marker for damage to these cells in vivo.
3.1 INTRODUCTION.

A marked creatinuria has been reported in rats subsequent to ligation of the
testicular vasculature, and exposure to two testicular toxicants, cadmium and 2-ME.\cite{332,335} The testis has been confirmed as the source of creatine after both cadmium and 2-ME intoxication, since cadmium did not elevate creatine levels in the urine of orchidectomised male rats, and neither cadmium nor 2-ME elevated urinary creatine when administered to female rats.\cite{333,335} Cadmium damages the testicular vasculature,\cite{2} resulting in an extensive, non-specific necrosis of all cell types within the organ.\cite{110,113} 2-ME is more selective, specifically destroying early and late stage pachytene primary spermatocytes.\cite{39,43,44}

MAA is an \textit{in vivo} oxidation product of 2-ME,\cite{45,48,49} that induces the same cell-specific lesion as the parent glycol ether.\cite{50,51} MAA, or a further metabolite, is considered to be the ultimate toxicant responsible for the testicular lesion induced by 2-ME,\cite{40,50} and metabolism of 2-ME to MAA has been shown to be a prerequisite for the lesion to be expressed.\cite{45,53} The toxicity of MAA is the result of a direct action upon the spermatocytes since it causes degeneration of these cells when incubated in a defined medium in the absence of Sertoli cells.\cite{57}

The testicular toxic phthalic acid esters (including DPP) and 1,3-DNB initially damage the Sertoli cells, with germ cell degeneration and exfoliation occurring as a secondary event within 24 h after dosing.\cite{63,64,74,85} The cellular targeting of these toxicants is reflected by alterations in the morphological and functional characteristics of Sertoli cells. These have been demonstrated both \textit{in vivo}, after the administration of the phthalate diesters or 1,3-DNB,\cite{67,68,73,85,362} and \textit{in vitro}, after the exposure of cultured Sertoli cells to the phthalate monoesters or 1,3-DNB.\cite{53,56,74,76,78,91,92}

The Leydig cell population is the only cell type to be affected up to 48 h after the administration of a single dose of EDS, although disruption and degeneration of the seminiferous epithelium occurs within a week, probably as a result of testosterone withdrawal.\cite{107,109} The effects of EDS are due to direct cytotoxic action upon the Leydig cells.
3.2 METHODS.

3.2.1 Animals.

Animals were housed and cared for as described (§2.4.1). Those treated with MAA, DPP, and 1,3- and 1,4-DNB were 4 weeks of age at the start of the experiment, since immature (smaller) rats are easier to handle and, in the case of the phthalate esters at least, are more sensitive to the induction of testicular damage than mature rats. Animals treated with EDS were 9 weeks old since EDS does not affect the immature rat testis.

3.2.2 Urine collection and assay.

Urine was collected over ice and stored at -20°C until assayed (§2.4.2). Creatine and creatinine were determined by the enzymatic method of Siedel et al as described (§2.6.1). High creatine values were confirmed by HPLC (§2.6.2).

3.2.3 Dosing of animals.

i) Dose-response experiments. Animals were randomly assigned to dosing groups. Following acclimatisation, they were administered single doses of MAA, DPP, 1,3-DNB, or EDS, according to the dosing regimens given in Table 3.1. In each experiment, control groups were given the respective vehicle alone.

ii) Comparison of 1,3- and 1,4-DNB. Animals were randomly assigned to dosing groups, and allowed to acclimatise, before administration of a single dose of either 1,3- or 1,4-DNB (50 mg/kg in 1.5% dimethylsulphoxide (DMSO) in corn oil, 5 ml/kg, p.o.). A control group was given the vehicle alone.

---

1 Polyethylene glycol, which has been used as a vehicle for the administration of DNB isomers to rats in a previous study, was found to induce a substantial increase in urinary creatine excretion (data not shown). 1.5% DMSO in corn oil was used as an alternative.
rather than as an indirect response to alterations in the testicular-hypothalamic axis, since specific functional and morphological changes are induced in cultured Leydig cells.\textsuperscript{106}

(A) \( \text{CH}_3\text{O-CH}_2\text{COO}^- \text{Na}^+ \)

(B) \[
\begin{array}{c}
\text{CO-OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3 \\
\text{CO-OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3 
\end{array}
\]

(C) i) \( \text{NO}_2 \) ii) \( \text{NO}_2 \)

(D) \[
\begin{array}{c}
\text{H}_2\text{CO-S-OCH}_2\text{CH}_2\text{O-S-OCH}_3 \\
\uparrow \\
\text{H}_2\text{CO-S-OCH}_2\text{CH}_2\text{O-S-OCH}_3 
\end{array}
\]

Figure 3.1. The structures of toxicants studied for their effects upon creatine excretion in the rat. (A) MAA (sodium salt), (B) DPP, (C) (i) 1,3-DNB and (ii) 1,4-DNB, (D) EDS.
Table 3.1 Dosing of animals in dose-response experiments. Sterile dosing solutions were prepared and administered in accordance with the following regimens.

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>Doses Administered (mg/kg)</th>
<th>Vehicle</th>
<th>Volume (ml/kg)</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAA</td>
<td>60, 300, 600, 900</td>
<td>0.9% saline (final pH 7.4)</td>
<td>2.2</td>
<td>i.p.</td>
</tr>
<tr>
<td>DPP</td>
<td>220, 440, 1100, 2200</td>
<td>corn oil</td>
<td>5.0</td>
<td>p.o.*</td>
</tr>
<tr>
<td>1,3-DNB</td>
<td>11.7, 29.4, 58.7</td>
<td>1.5% DMSO in corn oil</td>
<td>5.0</td>
<td>p.o.*</td>
</tr>
<tr>
<td>EDS</td>
<td>25, 50, 75</td>
<td>25% DMSO in water</td>
<td>2.0</td>
<td>i.p.</td>
</tr>
</tbody>
</table>

* By gavage.

3.2.4 Measurement of testicular and hepatic creatine.

Four to five week old rats were killed by cervical dislocation and the testes and livers rapidly excised and weighed. The left testis was placed in Bouin's fixative prior to preparation for histological examination. The right testis was weighed, decapsulated and placed on ice in a 14 × 100 mm plastic test-tube containing 4 volumes of phosphate buffer (0.1 M, pH 6.5). A portion of liver from a region that was free of major blood vessels was removed, weighed and placed on ice in 4 volumes of buffer as described. The tissues were homogenised on ice with two 2 s pulses with a Polytron homogeniser (Kinematica GmbH, Lucerne, Switzerland) set at power level 7.

PCr in the samples was converted to creatine by incubation with creatine kinase and ADP.\(^ {285} \) Half of a volume each of two reagents (0.1 M phosphate buffer, pH 6.5, containing 3000 U/ml creatine kinase; and 0.1 M phosphate buffer, pH 6.5, containing 20 mM ADP, 220 mM N-acetyl-L-cysteine, 166 mM magnesium acetate and 28 mM EDTA) were added, mixed, and the samples incubated at 37 °C for 1 h. They were then boiled for 5 min, cooled on ice and centrifuged at 2700 g for 5 min. Aliquots of each supernatant (0.5 ml) were diluted with 4.5 ml phosphate buffer (0.1 M, pH 8.0) prior to assay as described (§2.7.1).
3.3 RESULTS.

3.3.1 Urinary metabolite profiles and testicular structure of immature and mature control animals.

3.3.1a The excretion of creatine and creatinine.

Four week old control rats excreted creatine and creatinine in a ratio (Cr: Crn) of approximately 1:16 (1-2 and 20-30 μmol/24 h respectively) as shown in Tables 3.2-3.4 & 3.6. Nine week old rats excreted a similar amount of creatine, but very much more creatinine (55-65 μmol/24 h), and so the molar ratio was reduced to 1:48 (Figure 3.9, Table 3.5). This is because creatinine excretion is related to muscle mass. When creatinine excretion was expressed with respect to body weight, then there was no significant difference between the two age groups (data not shown).

3.3.1b Testicular histology.

Four week old rats all had a similar testicular histology. The testes were immature, with the most advanced germ cell types being spermatocytes and round spermatids. There were no elongating or maturation spermatids present (Figure 3.2a). Figure 3.2b shows a section of testis from a 9 week old rat displaying full spermatogenesis.
Figure 3.2. The structure of the rat testis. Histological preparations of testes from (A) an immature rat and (B) a mature rat. The various cell types are indicated as follows: Sg, spermatogonium; PPS, pre-pachytene spermatocyte; PS, pachytene spermatocyte; RS, round spermatid; ES, elongating spermatid; LC, Leydig cell. H&E, magnification x640.
3.3.2 The effects of MAA administration upon urinary excretion profiles, and pathological correlations.

3.3.2a The excretion of creatine and creatinine.

The levels of creatine and creatinine in the urine of animals treated with MAA are shown in Table 3.2. MAA treatment increased urinary creatine excretion at all doses, and a decrease in creatinine at 600 and 900 mg/kg, within the first 24 h period after dosing. These values had returned to pretreatment values before the next 24 h period. At no dose level did MAA affect the volume of urine excreted. When the excretion of creatine was expressed in relation to that of creatinine (Cr:Crn), MAA caused a dose-related creatinuria that was significantly different from control values in all but the lowest dose group (Figure 3.3).

![Figure 3.3](image)

Figure 3.3. The effect of MAA upon the relative excretion of creatine and creatinine, and upon relative testis weight in immature rats. The effect of MAA on Cr:Crn at t = 24 h (open circles) and on relative testis weight at t = 48 h (closed circles). Each point represents mean ± SEM (for n see Table 3.2). * Significantly different from control (P <0.05, Student's t-test).

3.3.2b The effects of MAA upon the testis.

i) Testis weight. MAA caused a decrease in relative testis weight, but only at 900 mg/kg was this statistically significant (Figure 3.3). MAA had no effect upon body weights (data not shown).
Table 3.2. The excretion of creatine and creatinine in the urine of rats after the administration of MAA. Urine was collected for 24 h prior to the administration of various single doses of MAA. Further urine collections were made during the two subsequent 24 h periods, and the excretion of creatine and creatinine compared to that during the pretreatment period.

<table>
<thead>
<tr>
<th>Dose MAA (mg/kg)</th>
<th>Creatine Excretion ( ^a ) (μmol/24 h)</th>
<th>Creatinine Excretion ( ^a ) (μmol/24 h)</th>
<th>( n^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time Period (h)( ^c )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-24 - 0</td>
<td>0 - 24</td>
<td>24 - 48</td>
</tr>
<tr>
<td>0</td>
<td>1.91 ± 0.46</td>
<td>1.22 ± 0.38</td>
<td>1.07 ± 0.15</td>
</tr>
<tr>
<td>60</td>
<td>3.13 ± 0.53</td>
<td>4.12 ± 1.14</td>
<td>2.06 ± 0.38</td>
</tr>
<tr>
<td>300</td>
<td>2.36 ± 0.23</td>
<td>8.39 ± 0.46( ^d )</td>
<td>3.05 ± 1.07</td>
</tr>
<tr>
<td>600</td>
<td>1.14 ± 0.31</td>
<td>5.72 ± 1.30( ^d )</td>
<td>2.14 ± 0.76</td>
</tr>
<tr>
<td>900</td>
<td>2.52 ± 0.46</td>
<td>11.75 ± 1.14( ^d )</td>
<td>3.59 ± 0.38</td>
</tr>
</tbody>
</table>

\( ^a \) Data are mean ± SEM
\( ^b \) Number of animals in each group
\( ^c \) Time 0 h = dosing
\( ^d \) Significantly different from the pretreatment value for the same group (\( P < 0.05 \), Student's t-test)
ii) Histology. When examined by light microscopy, the lowest dose of MAA (60 mg/kg) had no apparent effect upon the testis. At 300 mg/kg cell loss and necrosis was restricted to early pachytene spermatocytes at stages I and II and late and dividing spermatocytes at stages XIII and XIV, with 10% of the tubules at these stages being affected. At 600 mg/kg the damage had extended to include late pachytene spermatocytes at stage XII. At the highest dose employed (900 mg/kg) a moderate to marked loss of early spermatocytes at stages I and II, with some damage extending to stage VI, and of late and dividing spermatocytes at stages IX-XIV was noted (Figure 3.4).

Figure 3.4. The effect of MAA upon the histology of the testis. A section of immature rat testis 48 h after the administration of 900 mg/kg MAA. Note the loss of pachytene spermatocytes from the tubules (arrow heads). Other tubules remain unaffected. H&E, magnification ×640.
3.3.3 The effects of DPP administration upon urinary excretion profiles, and pathological correlations.

3.3.1a The excretion of creatine and creatinine.

DPP did not cause any alteration in urinary creatinine but had a pronounced effect upon creatine at the highest dose employed (Figure 3.5, Table 3.3). In the 24h period following the administration of 2.2 g/kg DPP, the level of creatine in the urine and Cr:Crn were significantly greater than the pretreatment value but had returned to normal by 48 h.

Although creatine excretion was increased after the administration of 220 mg/kg DPP, at no time was the excretion in this group statistically significantly different from that in the control group.

3.3.3b The effects of DPP upon the testis.

i) Testis weight. DPP induced a reduction in relative testis weight which was statistically significant at 1.1 and 2.2 g/kg (Figure 3.5). DPP had no effect upon body weights (data not shown).
Table 3.3. The excretion of creatine and creatinine in the urine of rats after the administration of DPP. Urine was collected for 24 h prior to the administration of various single doses of DPP. Further urine collections were made during the two subsequent 24 h periods, and the excretion of creatine and creatinine compared to that during the pretreatment period.

<table>
<thead>
<tr>
<th>Dose DPP (mg/kg)</th>
<th>Creatine Excretion * (μmol/24 h)</th>
<th>Creatinine Excretion * (μmol/24 h)</th>
<th>n^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time Period (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-24 - 0</td>
<td>0 - 24</td>
<td>24 - 48</td>
</tr>
<tr>
<td>0</td>
<td>1.37 ± 0.23</td>
<td>0.76 ± 0.31</td>
<td>1.07 ± 0.23</td>
</tr>
<tr>
<td>220</td>
<td>1.52 ± 0.15</td>
<td>0.53 ± 0.15</td>
<td>0.84 ± 0.00^d</td>
</tr>
<tr>
<td>440</td>
<td>1.22 ± 0.38</td>
<td>1.45 ± 0.15</td>
<td>1.07 ± 0.23</td>
</tr>
<tr>
<td>1100</td>
<td>0.53 ± 0.08</td>
<td>0.38 ± 0.15</td>
<td>0.38 ± 0.08</td>
</tr>
<tr>
<td>2200</td>
<td>2.21 ± 0.53</td>
<td>13.81 ± 2.52^d</td>
<td>2.59 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>19.1 ± 0.8</td>
<td>17.6 ± 1.0</td>
<td>17.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>18.8 ± 2.6</td>
<td>13.5 ± 1.3</td>
<td>14.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>18.3 ± 3.5</td>
<td>22.2 ± 1.3</td>
<td>21.9 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>15.5 ± 2.4</td>
<td>17.6 ± 1.3</td>
<td>12.5 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>22.6 ± 5.7</td>
<td>22.9 ± 2.4</td>
<td>15.7 ± 1.1</td>
</tr>
</tbody>
</table>

^a Data are mean ± SEM.

^b Number of animals in each group.

^c Time 0 h = dosing.

^d Significantly different from the pretreatment value for the same group (P < 0.05, Student's t-test).
ii) Histology. At a dose of 220 mg/kg, DPP had no effect upon the testis when examined by light microscopy. At 440 mg/kg and 1.1 g/kg, DPP caused slight to moderate exfoliation of late pachytene spermatocytes and round spermatids into the lumen of the tubules, but there was no germ cell necrosis and no obvious changes in Sertoli cell morphology. Extensive exfoliation of late pachytene spermatocytes and round spermatids from 90% of tubules was seen at 2.2 g/kg DPP, with some loss of prepachytene spermatocytes also. There was still no necrosis of germ cells nor alterations in Sertoli cells observed (Figure 3.6).

Figure 3.6. The effect of DPP upon the histology of the testis. A section of immature rat testis 48 h after the administration of 2 g/kg DPP. Note the extensive sloughing of germ cells from the epithelium into the lumens of the tubules (arrow heads). H&E, magnification x640.
3.3.4 The effects of 1,3-DNB administration upon urinary excretion profiles, and pathological correlations.

3.3.4a The excretion of creatine and creatinine.

Treatment with 1,3-DNB resulted in an increase in the 24 h urinary creatine excretion and Cr:Crn which was significant at both 30 and 60 mg/kg (Figure 3.7, Table 3.4). Urinary creatine levels had returned to pretreatment values within 24h after dosing, since creatine excretion in the 24-48 h period was the same as that prior to the administration of 1,3-DNB.

![Figure 3.7. The effect of 1,3-DNB upon the relative excretion of creatine and creatinine, and upon relative testis weight in immature rats.](image)

**Figure 3.7.** The effect of 1,3-DNB upon the relative excretion of creatine and creatinine, and upon relative testis weight in immature rats. The effect of 1,3-DNB on Cr:Crn at t = 24 h (open circles) and on relative testis weight at t = 48 h (closed circles). Each point represents mean ± SEM (for n see Table 3.4). * Significantly different from control (P <0.05, Student's t-test).

3.3.4b The effects of 1,3-DNB upon the testis.

i) Testis weight. 1,3-DNB caused a dose-related reduction in relative testis weight which was statistically significant at all dose levels (Figure 3.7). Body weights were not affected by treatment (data not shown).
Table 3.4. The excretion of creatine and creatinine in the urine of rats after the administration of 1,3-DNB. Urine was collected for 24 h prior to the administration of various single doses of 1,3-DNB. Further urine collections were made during the two subsequent 24 h periods, and the excretion of creatine and creatinine compared to that during the pretreatment period.

<table>
<thead>
<tr>
<th>Dose 1,3-DNB (mg/kg)</th>
<th>Creatine Excretion (^a) (µmol/24 h)</th>
<th>Creatinine Excretion (^a) (µmol/24 h)</th>
<th>(n^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time Period (h) (^c)</td>
<td>Time Period (h) (^c)</td>
<td></td>
</tr>
<tr>
<td>-24 - 0</td>
<td>0 - 24</td>
<td>24 - 48</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.68 ± 0.23</td>
<td>1.45 ± 0.23</td>
<td>21.7 ± 1.2</td>
</tr>
<tr>
<td>12</td>
<td>3.13 ± 0.99</td>
<td>1.68 ± 0.53</td>
<td>28.9 ± 3.3</td>
</tr>
<tr>
<td>30</td>
<td>2.29 ± 0.53</td>
<td>3.13 ± 0.46</td>
<td>20.8 ± 2.3</td>
</tr>
<tr>
<td>60</td>
<td>0.84 ± 0.31</td>
<td>8.39 ± 2.21 (^d)</td>
<td>30.6 ± 2.3</td>
</tr>
</tbody>
</table>

\(^a\) Data are mean ± SEM
\(^b\) Number of animals in each group
\(^c\) Time 0 h = dosing
\(^d\) Significantly different from the pretreatment value for the same group (\(P < 0.05\), Student's t-test)
ii) Histology. At the lowest dose (12 mg/kg) 1,3-DNB had no effect upon the testis but caused degeneration and depletion of a proportion of the late pachytene spermatocytes, which were phagocytosed or exfoliated, at 30 mg/kg. Pre-pachytene spermatocytes and round spermatids appeared unaffected. At 60 mg/kg 1,3-DNB almost all pachytene spermatocytes and round spermatids were found to be absent or degenerate, and multinucleate giant cells were observed (Figure 3.8).

Figure 3.8. The effect of 1,3-DNB upon the histology of the testis. A section of immature rat testis 48 h after the administration of 60 mg/kg 1,3-DNB. Note the extensive sloughing of germ cells from the epithelium into the lumens of the tubules (arrow heads). H&E, magnification ×640.
3.3.5 The effect of EDS administration upon urinary excretion profiles, and pathological correlations.

3.3.5a The excretion of creatine and creatinine.

Creatine excretion was elevated in all groups in the first 24 h after dosing, but there was no significant difference between that in the EDS treatment groups that in the control group (Table 3.5). Cr:Crn was not altered over the first 24 h in any of the groups (Figure 3.9). The excretion of both creatine and creatinine in the top dose group was substantially elevated in the first 24 h after dosing, although only in the case of creatinine excretion is this increase statistically significant (Table 3.5).

The concentrations of creatine and creatinine in the urine were not raised above control levels (data not shown), rather the results were due to due to extraordinarily high urine volumes from two of the four animals (32 ml and 64 ml, as opposed to 10 ml for the other two animals in the group, and 7-10 ml generally for the animals from other groups). The high urine volumes were accompanied by an apparently higher (50 ml) water consumption. It is possible that the higher creatine and creatinine excretion observed may
Table 3.5. The excretion of creatine and creatinine in the urine of rats after the administration of EDS. Urine was collected for 24 h prior to the administration of various single doses of EDS. Further urine collections were made during the two subsequent 24 h periods, and the excretion of creatine and creatinine compared to that during the pretreatment period.

<table>
<thead>
<tr>
<th>Dose EDS (mg/kg)</th>
<th>Creatine Excretion$^a$ (μmol/24 h)</th>
<th>Creatinine Excretion$^a$ (μmol/24 h)</th>
<th>$n^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time Period (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-24 - 0</td>
<td>0 - 24</td>
<td>24 - 48</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.53 ± 0.08</td>
<td>1.52 ± 0.15$^d$</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>0.38 ± 0.08</td>
<td>1.98 ± 0.15$^d$</td>
<td>4</td>
</tr>
<tr>
<td>50</td>
<td>0.76 ± 0.15</td>
<td>1.99 ± 0.69$^d$</td>
<td>4</td>
</tr>
<tr>
<td>75</td>
<td>1.06 ± 0.15</td>
<td>5.03 ± 1.91$^d$</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.52 ± 0.31$^d$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>64.8 ± 9.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.3 ± 12.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>68.4 ± 8.2</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Data are mean ± SEM
$^b$ Number of animals in each group
$^c$ Time 0 h = dosing
$^d$ Significantly different from the pretreatment value for the same group ($P < 0.05$, Student's t-test)
reflect an effect of water overload on the kidneys, or a reduced influence of absorption by food debris as urine volume increases. The relationship between urine volume and the measured excretion of urinary metabolites is shown in Figure 3.10.

![Figure 3.10. The relationship between urine volume and the measured excretion of creatine and creatinine. Data represent the 0-24 h urine collection for all animals in the study, represented as follows: controls, open circles; 25 mg/kg EDS, closed circles; 50 mg/kg EDS, open triangles; 75 mg/kg EDS, closed triangles.

3.3.5b The effects of EDS upon the testis.

i) Testis weight. EDS significantly reduced relative testis weight at both 50 and 75 mg/kg (Figure 3.9). Body weight was significantly reduced \( P < 0.05 \) at the highest dose (0.9 ± 1.2% loss, compared to a 2.7 ± 0.4% gain in the control group) but was not affected at the other two doses (data not shown).
ii) Histology. EDS at 25 mg/kg had no discernible effect upon testicular histology but at 50 mg/kg, although there was no change in the germinal epithelium, a proportion of the Leydig cells showed nuclear vesiculation, karyolysis and occasional pyknosis, and their normal rounded contour had given way to a more uneven, 'ragged' appearance. A slight mononuclear inflammatory infiltrate was also observed. In the 75 mg/kg EDS treatment group the Leydig cells had largely disappeared or were pyknotic, and there was a more marked mononuclear leucocyte infiltrate to that seen at 50 mg/kg, but the seminiferous epithelium was not affected and spermatogenesis appeared normal (Figure 3.11).

Figure 3.11. The effect of EDS upon the histology of the testis. A section of mature rat testis 48 h after the administration of 75 mg/kg EDS. Note the degenerate morphology of the few remaining Leydig cells (arrow heads). H&E, magnification ×640.
3.3.6 Comparison of the effects of two isomers of DNB upon urinary excretion
profiles, with pathological correlations.

3.3.6a The excretion of creatine and creatinine.

There was a decline in the excretion of creatine and creatinine in the control group
over the time course studied (Table 3.6), which may be due to the animals initially being
unsettled.

1,3-DNB caused an increase in the excretion of creatine within 24 h, but excretion
was not significantly different from pretreatment values after a further 24 h. 1,4-DNB
increased the excretion of creatine, but this effect was not statistically significant. Creatinine
excretion fell in both treatment groups but it is unclear as to whether this can be ascribed to
the treatments given.

3.3.6b The effects of 1,3-DNB and 1,4-DNB upon the testis and spleen.

i) Testis weight. 1,3-DNB significantly decreased relative testis weight to 0.71 ± 0.02 %
(mean ± SEM), but 1,4-DNB had no significant effect when compared to the control group
(0.79 ± 0.02 % and 0.81 ± 0.02 % respectively, Student's t-test, \( P < 0.05 \)). Body weight was
not affected by either treatment (data not shown).

ii) Spleen weight. Both 1,3- and 1,4-DNB significantly increased relative spleen weight
compared to the control group (0.67 ± 0.03 %, 0.56 ± 0.03 %, and 0.46 ± 0.02 %
respectively, mean ± SEM, Student's t-test, \( P < 0.05 \)).

iii) Testicular histology. 1,3-DNB caused a depletion of the seminiferous epithelium as
described previously (section 3.3.4bii), 1,4-DNB had no effect (not shown).

iv) Splenic histology. Both isomers caused a darkening of the spleen and an increase in the
red pulp, but had no effect upon the white pulp (Figure 3.12).
Table 3.6. Comparison of the effects of 1,3- and 1,4-DNB upon the excretion of creatine and creatinine. Urine was collected for 24 h prior to the administration of single doses (50 mg/kg) of 1,3- or 1,4-DNB. Further urine collections were made during the two subsequent 24 h periods, and the excretion of creatine and creatinine compared to that during the pretreatment period.

<table>
<thead>
<tr>
<th>Time Period (h)</th>
<th>Creatine Excretion(^a) (μmol/24 h)</th>
<th>Creatinine Excretion(^a) (μmol/24 h)</th>
<th>n(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-24 - 0</td>
<td>control: 5.82 ± 1.16</td>
<td>24.3 ± 1.9</td>
<td>5</td>
</tr>
<tr>
<td>0 - 24</td>
<td>1,3-DNB: 6.90 ± 1.66</td>
<td>28.7 ± 1.7</td>
<td>5</td>
</tr>
<tr>
<td>24 - 48</td>
<td>1,4-DNB: 7.11 ± 0.76</td>
<td>28.6 ± 2.0</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) Data are mean ± SEM
\(^b\) Number of animals in each group
\(^c\) Time 0 h = dosing
\(^d\) Significantly different from the pretreatment value for the same group (P < 0.05, Student's t-test)
Figure 3.12. The effect of 1,3-DNB upon the spleen. The photographs show sections of spleens from a control rat (A), and a rat treated with 50 mg/kg 1,3-DNB (B). Note the enlargement of the red pulp areas (RP), without any apparent effect upon the white pulp areas (WP). Similar effects were noted in the case of animals treated with 50 mg/kg 1,4-DNB (not shown). (H&E, magnification x640).
3.4 DISCUSSION.

3.4.1 Elevated creatinuria after testicular damage.

3.4.1a Correlations between creatine excretion and testicular damage after the selective destruction of testicular cells.

2-ME has been shown, both in vivo and in vitro, to require metabolic activation in order to express testicular toxicity, and administration of MAA, an oxidation product of 2-ME in vivo, results in a lesion that follows a pattern similar to that induced by its parent glycol ether. Selective germ cell degeneration occurs within 24 h of a single dose of MAA, and even after 48 h these were the only cell type affected in this study (Figure 3.4).

The loss in relative testis weight, and the histological findings, indicate that the extent of germ cell damage caused by MAA is reflected by the marked increase in urinary creatine excretion (Figure 3.4, Table 3.2). Moreover, whilst the excretion of creatine was elevated at all doses that induced histological changes, relative testis weight was only substantially, and statistically significantly, reduced at the highest dose employed. Therefore, in the case of MAA administration, creatinuria appears to be a more sensitive index of damage than relative testis weight, and at least with regard to the dosing regimen employed in these studies, as sensitive as histological examination. The extratesticular toxicity of MAA closely resembles that of 2-ME, and since 2-ME-induced creatinuria is testis-specific, then it is not unreasonable to believe that this is the case for MAA also. That is, the creatinuria induced by MAA is solely a result of the testicular lesion.

Both DPP and 1,3-DNB caused creatinuria (Figures 3.5 & 3.7, Tables 3.3 & 3.4) within the time course reported in the literature for Sertoli cell damage. Germ cell necrosis was not observed in the histological studies after the administration of DPP, but necrotic cells may be exfoliated before 48 h, when the animals were necropsied. The extensive reduction in relative testis weight and germ cell exfoliation observed after the administration of 440 mg/kg and 1.1 g/kg DPP, without an accompanying elevation in urinary creatine levels may reflect an exfoliation of germ cells without necrosis, whereas 2.2 g/kg
DPP causes both necrosis and exfoliation. Similarly, the reduction in relative testis weight was more pronounced than creatinuria after administration of 1,3-DNB and this too may reflect a loss of germ cells without necrosis. However, because there is such a close inter-relationship between the germ cells and Sertoli cells, it is difficult to draw firm conclusions from the data presented in this Chapter alone.

Both DPP and 1,3-DNB have toxic effects outside the testis, and their contribution, if any, to the creatinuria elicited by these compounds must be considered. The major extratesticular site of phthalate ester toxicity is the liver, but studies in which hepatotoxic effects have been induced by these compounds have mainly used chronic dosing regimens, rather than the acute exposures employed in the study of testicular toxicity. In order to assess the possible contribution of hepatic and testicular creatine to the elevation in creatine excretion induced by DPP, the total creatine content of these organs in 4 week old animals was measured (Table 3.7). Administration of 2.2 g/kg DPP caused an increase in creatine excretion of 1.53 mg/24 h, and whereas this could easily be accounted for by the testicular creatine pool (3.15 mg/organ, giving a total testicular creatine pool of more than 6 mg/animal), the liver creatine pool is too small (0.89 mg/organ) for it to make a major contribution, without extensive damage to the organ. Since the phthalate esters do not have such a severe effect upon the liver, the influence of extratesticular creatine pools upon the

<table>
<thead>
<tr>
<th>Table 3.7. Testicular and hepatic creatine content in 4 week old Sprague-Dawley rats.</th>
<th>Rats of the same age as those which had been dosed with DPP were used to determine testicular and hepatic creatine pools. Data concerning creatine excretion are from Table 3.3.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular creatine content</td>
<td>Hepatic creatine content</td>
</tr>
<tr>
<td>(mg/organ)</td>
<td>(mg/organ)</td>
</tr>
<tr>
<td>3.15 (1.79-4.78)</td>
<td>0.89 (0.52-1.24)</td>
</tr>
</tbody>
</table>

Values are mean (range)
DPP-induced creatinuria is probably not significant.

Blackburn et al have studied the acute toxicities of 1,2- and 1,4-DNB as well as that of 1,3-DNB. 1,3-DNB induces cyanosis and splenic enlargement as well as testicular atrophy, whilst 1,4-DNB causes only cyanosis and splenic enlargement. 1,2-DNB was found to be without effect. This differential toxicity between the meta and para isomers of DNB has been exploited in the study of the contribution of extratesticular effects to 1,3-DNB-induced creatinuria. Both 1,3- and 1,4-DNB caused a darkening and enlargement of the spleen. These changes were characterised by an increase in the red pulp areas. The two isomers appeared to be equipotent in producing these alterations. The red pulp is involved in the erythrophagocytic activity of the spleen, and so an increase in the extent of these structures within the organ reflects an increase in haemolysis, possibly a result of the induced cyanosis. Because creatine has been reported to be a component of erythrocytes, it was considered that this erythrophagocytic activity may contribute to 1,3-DNB-induced elevations in creatinuria. However, such a contribution is unlikely since, although 1,4-DNB elevated urinary creatine output, the increase was not significantly greater than control levels, and was much less than the increase induced by 1,3-DNB (Table 3.6).

However, the germ cell population is known to affect functional parameters of the Sertoli cells, both in vivo and in vitro (Table 1.3). Alterations in Sertoli cell morphology occur specifically after 2-ME intoxication, and the functional parameters of these cells are altered by MAA treatment, both in vivo, and in vitro. Therefore the possibility of changes in tubular creatine homeostasis as a result of alterations in Sertoli cell function following germ cell removal cannot be ruled out, and the increase in creatine excretion observed after germ cell degeneration induced by MAA (and 2-ME) cannot be used alone as evidence for the presence of creatine within these cells. At sacrifice, the testes of animals treated with DPP or 1,3-DNB exhibited damage to the Sertoli cells, but secondary effects within the germ cell population were also noted. Therefore these data alone do not allow a firm conclusion on the creatine content of Sertoli cells to be drawn. Germ cells are intimately associated with
the Sertoli cells, and are highly dependent upon them for mechanical and metabolic support (§1.2). This is reflected by observations of secondary effects occurring within the germ cell population shortly after toxic insult to the Sertoli cells.\textsuperscript{53, 67, 68, 70, 85, 88, 91, 362} Although degenerative changes are observed in Sertoli cells only one hour after the administration of DPP to rats, extensive germ cell degeneration occurs within 24 h.\textsuperscript{67} Exposure of Sertoli-germ cell co-cultures to mono-n-pentyl phthalate, the putative toxic metabolite of DPP, results in altered Sertoli cell morphology and germ cell exfoliation also within 24 Similar observations have been reported for 1,3-DNB both \textit{in vivo} and \textit{in vitro}.\textsuperscript{85, 91} Therefore, the elevation in urinary creatine observed after the administration of Sertoli cell toxicants may well be to some extent a result of ensuing germ cell degeneration, and Sertoli cell damage \textit{per se} may not be the direct or only cause.

An added complication is, of course, the relative timing of the acme of creatine excretion and histological sampling. A more accurate assessment of the involvement of various cell (sub-)populations in the development of creatinuria could only be made if histological samples were obtained at the acme of creatine leakage from the testis, as determined from more frequent measurements of creatine concentrations in the urine, or even plasma.

Although EDS extensively depleted the Leydig cell population (Figure 3.11), and caused a marked loss in actual and relative testis weights (11\% and 8\% respectively in the 75 mg/kg dose group, \(P < 0.05\) with respect to controls), it did not cause a significant elevation in urinary creatine (Figure 3.9, Table 3.5). Although the excretion of creatine and creatinine was elevated in the 75 mg/kg group 24h after dosing, this effect was probably a result of water overload (§3.3.5a), and not due to a specific action upon the testis. When creatine excretion was expressed as a ratio to that of creatinine, EDS was without effect at all dose levels employed. Therefore it appears unlikely that creatine is associated with the Leydig cells. However, because of its proposed role in cytoskeletal movement, creatine may be associated with other interstitial cell types such as macrophages and fibroblasts.\textsuperscript{302, 317}
3.4.1b Possible mechanisms by which testicular damage could result in elevations in urinary creatine.

Because creatine is associated with cells of the seminiferous epithelium, then degeneration of these cells, either by direct cytotoxic action, or as a secondary effect after damage to other cells, may alter creatine storage or metabolism within the tubular compartment of the testis. Creatinuria arising as a result of damage to the seminiferous epithelium could result from the passage of creatine out of the testis, or a reduction in creatine uptake by the testis from the blood. Creatine may leave the tubular compartment of the testis by three possible mechanisms, by leakage across the blood-testis barrier into the interstitium; by uptake or diffusion from the epididymis; or by an alteration of Sertoli cell function. Alternatively, there may be a decrease in sequestration from the bloodstream.

i) Leakage of creatine across the blood-testis barrier. The blood-testis barrier is normally highly restrictive to the movement of chemicals between the tubular and the interstitial compartments, but leakage of creatine out of the tubular compartment is plausible, since the activity of LDH-X, a large (140 kD) germ cell-specific enzyme is elevated in the serum of animals treated with various testicular toxicants, and measurement of the activity of this enzyme in the serum has been suggested as a marker for testicular damage. Disruption of the integrity of the barrier would not have to be a prerequisite for creatine leakage, since creatine uptake into various cell types involves a component of passive diffusion. The ability of creatine to diffuse across biological membranes would allow it to exit the tubular compartment without any breakdown in the barrier architecture. Although the passive diffusion of creatine across biological membranes requires the maintenance of a high concentration gradient, removal of creatine from the interstitium, into the blood and urine, could allow extensive leakage from the tubular compartment of the testis, by preventing the establishment of an equilibrium across the blood-testis barrier (Figure 3.13).
Figure 3.13. The effect of creatine removal into the blood and urine upon its leakage from the tubular compartment of the testis. (A) As creatine crosses the blood-testis barrier (BTB), an equilibrium is established and net efflux ceases. (B) Removal of creatine from the interstitium into the blood and urine prevents the establishment of an equilibrium across the BTB, and thus facilitates diffusion from the tubular compartment. Since the volume of the blood and urine can be considered to be infinite in comparison to that of the interstitium, then equilibrium is not established between these two compartments, and thus creatine diffusion out of the testis is maintained.

**ii) Leakage of testicular creatine via the epididymis.** Sertoli cells secrete fluid into the adluminal compartment of the testis and, as this fluid leaves the organ, 96% is reabsorbed between the rete testis and the cauda epididymis. If germ cell or Sertoli cell damage was to result in an increase in the concentration of creatine in the tubular fluid, then creatine could pass into the bloodstream via absorption by the epididymis. However, this route for creatine leakage may be less likely, at least in the case of DPP-induced creatinuria, since DPP completely inhibits Sertoli cell fluid secretion within one hour of the administration of a single dose of 2.2 g/kg, continuing over at least 17 h. This could exclude the epididymis as a route of creatine leakage out of the testis after DPP-induced damage. 1,3-DNB also reduces tubular fluid secretion by Sertoli cells within 3 h of the administration of a single dose of 32 mg/kg, although the effect is not as pronounced as for DPP (approximately 35% reduction), and returns to a level significantly higher than control within 24 h.
iii) Creatine release as a result of alterations in Sertoli cell homeostasis. A wide range of products are secreted by Sertoli cells. These secretory functions can be altered by the presence or absence of germ cells (§1.2.2bi) and by the action of Sertoli cell toxicants (Table 5.1). Although cultured Sertoli cells secrete creatine into the incubation medium (Chapters 4 & 5), there is no evidence that a directional secretion of creatine occurs in vivo, or that an alteration of such secretion could account for the elevation in urinary creatine observed. Such a mechanism cannot be ruled out without further investigations, but it is unlikely to be a major contributing factor when the transient nature of the induced creatinuria is considered.

Urinary creatine excretion was elevated and returned to normal within 24 h of the administration of effective doses of MAA, DPP and 1,3-DNB, since excretion within the 0-24 h period was greater than that prior to treatment, but there was no difference in excretion between the pretreatment and 24-48 h post treatment periods. The increase in urinary creatine excretion is therefore only transitory, indicative of an 'all or none' effect (e.g. cell lysis). In marked contrast to this are the reports in the literature concerning the long-term nature of alterations in Sertoli cell function, induced both by the direct action of cytotoxicants, and as a result of disturbance to the germ cell population. Levels of FSH in the serum and ABP in the testicular interstitial fluid were elevated for at least three days after MAA administration. After DPP administration, ABP secretion into the interstitium was elevated within two days, and remained above control levels for three weeks, whilst 1,3-DNB treatment caused an elevation in seminiferous tubular fluid ABP levels within 24 h which remained above control for one week, and elevations in serum and testicular interstitial fluid ABP levels within one week, which remained above control for two weeks. If creatinuria was caused by alterations in Sertoli cell secretory functions, then the elevations observed might be expected to be of much greater duration.

iv) Increased creatine excretion as a result of decreased uptake from the blood. There is only limited support in the literature for a creatine uptake system in the testis.
Hyperthyroidism in the rat is associated with increases in plasma (and urinary) creatine concentrations, as creatine is lost from muscle stores. Treatment of rats with thyroid extracts and thyroxine resulted in a decrease in creatine levels in skeletal and cardiac muscle (by 9-19% and 43-49% respectively) but an increase in testicular creatine levels (by 5-11%). Presumably an elevation in plasma creatine concentration allows a greater uptake of creatine into the testis.

Although cellular degeneration could result in a reduction in any uptake that might occur, as with other hypotheses implicating changes in creatine homeostasis following alterations in cellular function it is unlikely to account for the observations reported in this Chapter. The elevations in urinary creatine excretion elicited by MAA, DPP and 1,3-DNB are only transient, with excretion returning to pretreatment values within 24 h. If increases in creatine excretion were due to decreased uptake by the testis, then a more prolonged creatinuria would be expected, only returning to normal as the seminiferous epithelium became repopulated. Such repopulation does not occur within the time course of the experiments reported here, as evidenced by the accompanying histological studies and the published data concerning the time course of spermatogenesis.

Published data concerning creatinuria in other toxic or diseased states suggest that transient elevations are due to loss from cellular storage sites as necrosis occurs, whilst prolonged elevations are a result of alterations in synthesis, uptake or retention by cellular stores. When the temporal profiles of MAA-, DPP-, and 1,3-DNB-induced creatinuria are compared with these observations, then it seems likely that creatinuria following testicular damage occurs as a result of leakage from necrotic or degenerating cells, rather than as a result of alterations in the normal metabolic pathways for creatine within the testis.

3.4.2 Creatinuria as a non-invasive marker for testicular damage.

A variety of indicators have been identified as markers of testicular damage. Biochemical markers include alterations in the levels of sex hormones, testicular proteins/
enzyme activities, and minerals/biochemical intermediates in the serum, urine or testis itself, whilst physiological markers include alterations in testicular histology, testis weight, and fertility of treated animals. These markers have been used to assess the course and extent of testicular lesions induced by a diversity of testicular toxicants including cadmium, 2-ME, 1,3-DNB, DPP, acrylamide, caffeine, procarbazine, fluoroacetic acid, 2,5-hexanedione and EDS.\textsuperscript{32 - 45, 49 - 51, 63 - 70, 79, 84 - 91, 93 - 105, 107 - 113, 171, 196, 230, 265, 267, 361, 365 - 367, 375 - 382} However, all of these markers involve some measure of invasion, even animal sacrifice, and will therefore cause varying degrees of disturbance within an experimental protocol. The use of daily sperm production or fertility assessment have further drawbacks, since when studying effects upon early stages in spermatogenesis they can only give indirect, delayed data as maturation depletion of late-stage spermatids or spermatozoa occurs.

Previous investigations into urinary creatine excretion after the administration of testicular toxicants have shown that a rapid elevation in excretion occurs, and that the extra creatine is derived from the testis.\textsuperscript{332 - 335} The data presented in this Chapter extend the range of xenobiotics to which creatinuria can be applied as a measure of cytotoxicity, define more clearly the cell types within the testis to which creatinuria could be relevant as a marker, and thus allow a broader evaluation of creatinuria as an index of testicular damage. The positive effects of MAA, DPP and 1,3-DNB, and the lack of effect of EDS, indicate that creatinuria will only allow damage to the seminiferous epithelium to be detected. However, deleterious effects to the Leydig cells result in secondary degenerative changes within the tubular compartment,\textsuperscript{108, 109} and such effects may possibly be monitored in this indirect manner. To resolve this further, it would be necessary to examine the effect of EDS over a longer time course (e.g. up to one week, when degeneration of the seminiferous epithelium occurs). Further experiments must also be carried out to determine how extensively creatinuria can be applied to the lesions of other known testicular toxicants and, particularly important for monitoring human exposure, whether or not creatinuria is related to testicular damage in man.
After acute exposure to toxicants that damage the seminiferous epithelium, there is a rapid and brief increase in the excretion of creatine in the urine (Figures 3.3, 3.5 & 3.6, Tables 3.2-3.4 & 3.6). Although this allows exposure and the course of ensuing degenerative changes to be monitored under laboratory conditions, in itself it would be of limited usefulness in the context of industrial, accidental, or environmental exposure. Under such conditions, the exposure of the individual to agents that damage the testis is often of a low level (sub-acutely toxic), long-term nature, and urine samples immediately after exposure may not be readily obtainable. Recent work carried out in this laboratory has shown that chronic exposure of rats to sub-acute toxic doses of 2-ME causes a prolonged elevation in urinary creatine excretion. These observations seem to support the validity of creatinuria as a possible index of testicular damage.

Because creatine is associated with many cell types and organs other than the testis, it is important to determine the contribution of extratesticular effects to the excretion of creatine. Although the contribution of erythrophagocytosis to 1,3-DNB-induced creatinuria appears minimal, other degenerative processes may produce a more significant interference. Elevated creatinuria has been correlated with a number of deviations from homeostasis including acute myocardial infarction, skeletal muscle trauma, muscular dystrophy, muscle wasting, hyperthyroidism, starvation, liver necrosis, bone fracture, vitamin E deficiency, adrenalectomy and the administration of diverse chemical agents such as pyrazole, methylated androgens and creatine analogues.

However, it would be possible to enhance the selectivity of creatinuria to testicular damage by coupling it with other diagnostic tests that are more specific to the testis, serum LDH-X activity for example. Because creatine is a much smaller molecule than LDH-X (molecular weights of 131 and 140,000 respectively), then it’s likely to escape

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1 M. Butterworth, J.A. Timbrell. Personal communication.
more rapidly from the testis into the blood, and hence urine. Therefore, monitoring of urinary 
creatine after testicular damage may allow more selective sampling of blood from laboratory 
animals (and perhaps humans), thus reducing the stress that they are subjected to by the 
use of invasive sampling procedures. This proposal is supported by reports that after the 
initial onset of symptoms of acute myocardial infarction, creatinuria precedes elevations in 
serum CPT activity by 5-15 h. This phenomenon has been explained by the vast difference 
in the molecular weights of the two markers, allowing creatine to escape from damaged 
cardiac tissue more rapidly than CPT.\textsuperscript{371}

The measurement of urinary metabolites allows non-invasive monitoring of disease 
or toxic stress. From the data presented here and elsewhere,\textsuperscript{332-335} it appears that 
creatinuria may provide an important measure of testicular damage, offering both a rapid and 
prolonged indication of degenerative changes within the organ. However, the potential 
contribution of other factors (such as cell interactions within the testis, and other sites of 
creatine metabolism within the body) must not be overlooked.

3.4.3 Further studies.

Further examinations of creatinuria following testicular damage should be made to 
further evaluate this marker.

a) The administration of drugs that directly affect the seminiferous epithelium and 
damage specific germ cell types (e.g. methyl methane sulphonate, hydroxyurea, 
vinka alkaloids, procarbazine etc.) and treatments that indirectly disturb 
spermatogenesis (e.g. hypophysectomy, EDS treatment) should be studied.

b) Correlations between creatinuria and other markers for testicular damage (e.g. 
serum LDH-X and FSH) should be evaluated.

c) In addition, in order to assess the relevance of the studies to man, the testicular 
creatine content in the human testis, and the excretion of creatine after exposure to 
testicular toxicants must be examined.
CHAPTER 4

THE CELLULAR DISTRIBUTION OF CREATINE AND OF THE ENZYMES INVOLVED IN ITS METABOLISM WITHIN THE SEMINIFEROUS EPITHELIUM
ABSTRACT.

Sertoli and germ cells were isolated from the testes of 4 week old rats and examined for their creatine content and for evidence of creatine metabolism. Both cell types were found to contain creatine and PCr pools, and to express CPT activity.

Isolated seminiferous tubules synthesised creatine, and its biosynthetic precursor, GAA, from L-arginine, as determined by radiolabel tracer studies using L-[guanidino-14C]arginine. A crude interstitial cell preparation did not synthesise either creatine or GAA.

Cultured Sertoli cells incorporated radioactivity from both L-[guanidino-14C]arginine and [1-14C]glycine into creatine and GAA. Therefore, Sertoli cells have the capacity to carry out both stages of creatine synthesis; transamidination between arginine and glycine, with subsequent methylation of GAA to creatine. Germ cells did not exhibit this activity.

From these observations it is concluded that, whilst only the Sertoli cells can synthesise creatine, both cell types have the capacity for its utilisation. Therefore the possibility of an intra- and intercellular creatine pathway within the seminiferous epithelium must be considered.
4.1 INTRODUCTION

The rat testis is recognised as a major site of creatine localisation, a proportion of which is found in the phosphorylated state. However, investigations into the distribution of creatine within the testis have not until now been carried out, although primary spermatocytes and spermatids have been reported to contain PCr.

The testis is unusual amongst those organs with a high creatine content in that it also synthesises creatine. This was first demonstrated in vitro when testicular tissue was incubated in the presence of [14C]glycine. The results were corroborated by in vivo experiments in which the testes of anaethetised rats were injected with [14C]arginine and [14C]GAA.

The purpose of the work described in this Chapter was to determine the testicular distribution of creatine and the enzymes associated with its metabolism.

4.2 METHODS.

4.2.1 The measurement of creatine and PCr, and CPT activity in cultured Sertoli cells and isolated germ cells.

SCEC prepared on 50 mm petri dishes were scraped into ice-cold saline (2 ml). Freshly isolated germ cells were pelleted (45 g, 5 min) and resuspended in ice-cold saline. The cells were homogenised on ice by two 5s pulses with a sonic homogeniser (MSE Soniprep 150) at a power setting of 20 μ, and then stored at -80 °C (samples for creatine and PCr assays) or 4 °C (samples for CPT and protein assays) prior to analysis as described (§2.7.1, §2.7.3, §2.7.4 & §2.9.1).

4.2.2 The histochemical localisation of CPT activity within the testis.

The distribution of CPT activity was determined by the linked enzyme method described by Frederiks et al.386

Principle. The basic principle behind this method is the same as that for the quantitative determination of CPT activity (§2.9.1). The enzyme activity is visualised within the tissue by the production of an insoluble, opaque formazan deposit formed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) by NADPH, with phenazine ethosulphate (PES) acting as an intermediary electron carrier:

\[
\text{NADPH} \rightarrow \text{PES}_{\text{ox}} \rightarrow \text{Formazan} \rightarrow \text{NADP}^+ \rightarrow \text{PES}_{\text{red}} \rightarrow \text{MTT}
\]

Reagents. Hexokinase (100 U) and glucose-6-phosphate dehydrogenase (48 U), dissolved in 0.6 ml distilled water; the incubation medium contained 17% polyvinyl alcohol (average molecular weight 40 kD), 0.1 M Tris (pH 7.4), 20 mM PCr, 10 mM MgCl₂, 10 mM D-glucose, 1.5 mM ADP, 4 mM AMP, 15 mM sodium azide, 0.45 mM NADP⁺, 0.2 mM PES,
5 mM MTT (a medium to measure 'background activity' was prepared in the same manner, but with the omission of PCr); 0.1 M phosphate buffer (pH 5.3), warmed to 60 °C; 4% formaldehyde in water.

**Method.** Microscope slides were cleaned overnight in 70% acid-alcohol prior to use. When the slides were dry, hexokinase/glucose-6-phosphate dehydrogenase solution (10 μl) was spotted onto each, spread over approximately 1 cm² with the pipette tip and allowed to dry.

The testes of a mature animal were removed, immediately frozen in liquid nitrogen-cooled 2-methylbutane (iso-propane), and mounted in preparation for sectioning. Sections of 8 μm thick were cut at -20 °C in a Reichert-Jung 2800 Frigocut N cryostat, and were positioned on the microscope slides such that the enzyme film was interposed between the tissue and the slide.

The incubation medium was placed on top of the tissue, and the samples were then left to incubate at room temperature for 10 min. After incubation, the medium was removed with warm phosphate buffer, the tissue postfixed in 4% formaldehyde, and then mounted in glycerol.

### 4.2.3 The metabolism of L-[guanidino-¹⁴C]arginine by isolated seminiferous tubules and interstitial cells.

Seminiferous tubules and interstitial cells were isolated as described (§2.5.2 & §2.5.3). L-[guanidino-¹⁴C]Arginine was added to the incubation medium to give a final isotope concentration of 0.1 μCi/ml. The tissue was incubated for 3 h in a shaking water bath (1.17 Hz) at 32 °C in a 5% CO₂/95% O₂ atmosphere.

At the end of the incubation the tissue samples were homogenised by sonication (two 5 s pulses at a setting of 20 μ), boiled for 5 min and centrifuged for 5 min at 2700 g. Aliquots (50 μl) of the supernatants were applied directly to the column.
4.2.4 The synthesis of creatine by SCEC and SGCC.

Random SGCC from two separate cell preparations were selected for germ cell removal. The resulting SCEC, and the remaining SGCC, were used after a further 24h.

SCEC and SGCC were scraped into the medium, either immediately after the replacement of the medium or after a further 24 h incubation. The cells were homogenised by sonication, and the creatine content determined by fluorometry (§2.8.2).

4.2.5 The metabolism of radiolabelled substrates by SCEC and isolated germ cells.

Twenty four hours after the removal of germ cells from SGCC, the medium was replaced with 2.5 ml fresh medium, containing L-[guanidino-14C]arginine or [1-14C]glycine added to a final activity of 0.1-0.2 μCi/ml. Freshly isolated germ cells were incubated in 35 mm petri dishes in 2.5 ml supplemented EMEM containing L-[guanidino-14C]arginine (0.1 μCi/ml).

SCEC were incubated for 24 h, after which time the medium was removed and centrifuged (45 g, 5 min), or the cells were scraped into the medium and homogenised by sonication (two 5 s pulses at a setting of 20 μ). The samples were then stored at 4 °C prior to treatment as described (§2.10.2).
4.3 RESULTS.

4.3.1 Creatine and PCr levels and CPT activity in isolated cells of the seminiferous epithelium.

The levels of creatine, PCr, and CPT activity in Sertoli cells and germ cells are given in Table 4.1. A small fraction (3-16%) of the total creatine content of both cell types was found in the phosphorylated state. The Sertoli cells appear to phosphorylate a greater fraction of their creatine, and this is reflected by a higher activity of CPT within these cells. However, the germ cell isolation method used in these studies only allowed a heterogenous population to be prepared, and so variations in the content and phosphorylation of creatine between subpopulations of germ cells cannot be determined.

The histochemical localisation of CPT is shown in Figure 4.1, and indicates activity within the seminiferous epithelium. The basal region of the epithelium is stained in sections that were incubated in the presence of PCr (Figure 4.1A & C), but not when PCr was omitted from the medium (Figure 4.1B & Figure 4.1D). This clearly indicates that such staining was dependent upon the presence of PCr, and is therefore due to the presence of CPT. Figure 4.1E is a section of rat testis, stained with H&E, showing the basal lamina region for comparison (cf. Figure 4.1C).

Table 4.1. The creatine, PCr and CPT content of Sertoli cells and germ cells.

<table>
<thead>
<tr>
<th></th>
<th>Creatine Content (nmol/mg protein)</th>
<th>PCr Content (nmol/mg protein)</th>
<th>CPT Activity* (mU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sertoli cells</td>
<td>90</td>
<td>17</td>
<td>8.8</td>
</tr>
<tr>
<td>Germ cells</td>
<td>260</td>
<td>9</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* One unit of CPT is defined as the activity required to dephosphorylate PCr at a rate of 1 μmol/min at a temperature of 32 °C.
Figure 4.1. The histochemical localisation of CPT activity within the rat testis. (A) & (C) show sections of testis incubated as described (§4.2.2) in the presence of PCr, while the 'background activity', or reagent blank (i.e. sections incubated in the absence of PCr), is shown in (B) & (D). Note that staining is more marked in those sections incubated in the presence of PCr. This difference indicates regions of CPT activity. Staining is particularly prominent around the basal region of the epithelium (arrows). (E) shows a section of testis, stained with H&E, detailing the region of the basal lamina. (A) & (B) - magnification ×160; (C), (D) & (E) - magnification ×640.
4.3.2 Creatine synthesis by isolated seminiferous tubules and interstitial cells.

Over a 3 h incubation period, seminiferous tubules synthesised both \(^{14}\text{C}\text{creatine}\) and \(^{14}\text{C}\text{GAA}\) from \(L-[\text{guanidino-}^{14}\text{C}]\text{arginine}\) (Figure 4.2, Table 4.2). The synthesis of GAA by seminiferous tubules isolated from immature rats was about 10\% that of creatine (Figure 4.2A), but was 70\% that of creatine in tubules from mature rats (Figure 4.2B).

A crude interstitial preparation did not significantly synthesise either metabolite.

Boiling the tubular tissue for 5 min prior to incubation destroyed its synthetic capacity.

---

Table 4.2. The synthesis of creatine and GAA by tubular and interstitial tissue isolated from immature and mature rats. Tissue was isolated from immature (4 weeks old) or mature (16-17 weeks old) rats, and incubated in HBSS containing \(L-[\text{guanidino-}^{14}\text{C}]\text{arginine}\) (0.08-0.10 \(\mu\text{Ci}/\text{ml}\)) for 3 h. The incorporation of radiolabel into soluble metabolites was determined by HPLC, and is expressed as the percentage of radioactivity loaded onto the column.

<table>
<thead>
<tr>
<th>Age of Rats (weeks)</th>
<th>Metabolite</th>
<th>Radioactivity Incorporated (% column loading) *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Seminiferous Tubules</td>
</tr>
<tr>
<td>4</td>
<td>GAA</td>
<td>1.9, 2.3</td>
</tr>
<tr>
<td></td>
<td>creatine</td>
<td>32.8, 16.1</td>
</tr>
<tr>
<td>16-17</td>
<td>GAA</td>
<td>13.0 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>creatine</td>
<td>18.1 ± 3.1</td>
</tr>
</tbody>
</table>

* Data are single, averaged values of 3-5 determinations from tissue preparations derived from individual animals, or mean ± SD from three animals.

b ND, not determined.
Figure 4.2. The incorporation of radioactivity from L-[guanidino-^{14}C]arginine into creatine by isolated rat seminiferous tubules. Tubules from (A) immature and (B) mature rats were incubated in the presence of L-[guanidino-^{14}C]arginine as described (§4.2.3). HPLC radiochromatograms show the incorporation of radioactivity into both GAA (peak I) and creatine (peak II).
4.3.3 The synthesis of creatine by SCEC and SGCC.

The total creatine content of SCEC and SGCC immediately after the replacement of the culture medium, and after a further 24 h incubation is given in Figure 4.3.

The synthesis of creatine by SCEC was 27 nmol/culture/24 h for one cell preparation, and 43 nmol/culture/24 h for the other. For both SGCC preparations, the synthesis of creatine was 43 nmol/culture/24 h.

Figure 4.3. The synthesis of creatine by SCEC and SGCC. The total creatine content of the cultures was determined immediately (0 h) or 24 h after the replacement of the medium. The increase in creatine content over this time is a measure of its synthesis. Data are the mean and range from two preparations of 3 or 4 dishes.
4.3.4 The metabolism of L-[guanidino-$^{14}$C]arginine by SCEC and cultured germ cells.

SCEC and isolated germ cells were incubated in the presence of L-[guanidino-$^{14}$C]arginine for 24 h, after which time the cells were scraped into the medium and the incorporation of radioactivity into metabolites was determined by HPLC. Figure 4.4 shows typical radiometabolic profiles for SCEC and germ cells.

SCEC incorporated radioactivity from arginine into both GAA and creatine (1.2 ± 0.1% and 1.7 ± 0.4%, mean ± SD from 4 culture dishes, equivalent to 17.3 and 24.8 nmol/culture/24 h respectively, Figure 4.4A).

Germ cells, cultured at a similar cell protein density to the Sertoli cells (0.254 and 0.303 mg protein/dish respectively), did not synthesise these metabolites (the small creatine peak, <10% of that produced by SCEC, was probably a result of contaminating Sertoli cells, which made up 7-9% of the cell population), although they still retained >98% viability (Figure 4.4B).
Figure 4.4. The Incorporation of radioactivity from L-[guanidine-14C]arginine into creatine by cultured Sertoli cells and isolated germ cells. SCEC (A) and isolated germ cells (B) were incubated in the presence of L-[guanidine-14C]arginine in EMEM (0.17 μCi/ml, final specific activity 0.28 μCi/μmol) for 24 h as described (§4.2.5). At the end of the incubation the medium was removed and applied to the HPLC column as described (§2.10). SCEC incorporated radioactivity into both GAA (peak I) and creatine (peak II), but germ cells expressed no significant activity. The shaded areas represent radiochromatograms of medium containing the radiolabelled precursor incubated without cells.
4.3.5 The secretion of L-[guanidino-\textsuperscript{13}C]arginine and [1-\textsuperscript{14}C]glycine metabolites by SCEC.

Radiometabolic profiles of media sampled from SCEC after 24 h incubations with either L-[guanidino-\textsuperscript{13}C]arginine or [1-\textsuperscript{14}C]glycine are shown in Figure 4.5. Both arginine and glycine were incorporated into GAA and creatine found in the culture medium (Figure 4.5, Table 4.3). Creatine accounted for 0.9% and 1.3% of the radioactivity in the media of SCEC exposed to L-[guanidino-\textsuperscript{13}C]arginine and [1-\textsuperscript{14}C]glycine respectively.

The final concentration of creatine in the medium was calculated from the incorporation of L-[guanidino-\textsuperscript{13}C]arginine to be $5.2 \pm 0.3 \mu M$, assuming no interference from endogenous sources.

Table 4.3. The incorporation of radioactivity from synthetic precursors into GAA and creatine. SCEC were incubated in EMEM with L-[guanidino-\textsuperscript{13}C]arginine (0.17 \mu Ci/ml, final specific activity 0.28 \mu Ci/\mu mol) or [1-\textsuperscript{14}C]glycine (0.15 \mu Ci/ml, final specific activity 1.5 \mu Ci/\mu mol) for 24 h. After this time the medium was removed and the radiometabolite profile examined by HPLC (Figure 4.4).

<table>
<thead>
<tr>
<th>Amount of Substrate Utilised (^a) (nmol)</th>
<th>Radiolabelled Substrate Incorporated per Culture (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAA (%), Creatine (%), GAA (nmol), Creatine (nmol)</td>
</tr>
<tr>
<td>Arginine 85.5 ± 9.8</td>
<td>0.6 ± 0.0, 0.9 ± 0.1, 9.0 ± 0.0, 13.0 ± 0.9</td>
</tr>
<tr>
<td>Glycine 20.9 ± 2.2</td>
<td>0.7 ± 0.1, 1.3 ± 0.1, 1.9 ± 0.1, 3.2 ± 0.1</td>
</tr>
</tbody>
</table>

\(^a\) Data are mean ± SD from three culture dishes.
Figure 4.5. The metabolism of L-[guanidino-\textsuperscript{14}C]arginine and [\textsuperscript{14}C]glycine by cultured Sertoli cells. SCEC were incubated for 24 h in EMEM containing either (A) L-[guanidino-\textsuperscript{14}C]arginine (0.17 μCi/ml, 0.28 μCi/μmol), or (B) [\textsuperscript{14}C]glycine (0.15 μCi/ml, 1.5 μCi/μmol). The media were collected and applied to the HPLC column as described (§2.10). Both precursors were incorporated into GAA (peak I) and creatine (peak II). The shaded areas represent radiochromatographic profiles of media containing the radiolabelled precursors and incubated in the absence of cells.
4.4 DISCUSSION.

4.4.1 The cellular distribution of creatine within the seminiferous epithelium.

The testis has substantial reserves of creatine, a proportion of which is found in the phosphorylated state.\textsuperscript{246-252, 327} The data presented in Chapter 3 indicate that creatine is associated with the seminiferous epithelium, but for reasons discussed in §3.4, the cellular distribution could not be further elucidated from \textit{in vivo} studies alone.

Isolation of the major cellular subpopulations of the seminiferous epithelium, the germ cells and the somatic Sertoli cells, has shown that both cell types contain creatine. The germ cell creatine content has been confirmed both by isolation of a mixed germ cell population (Table 4.1), and by the difference in creatine content between SCEC and SGCC (Figure 4.3). SCEC, prepared from the co-cultures by exhaustive removal of the germ cells with a hypotonic shock treatment, were used to demonstrate and measure the creatine content of Sertoli cells (Table 4.1, Figure 4.3).

Both Sertoli cells and germ cells were found to express CRT activity and to phosphorylate a proportion of their creatine pool (Table 4.1). The PCr content, in relation to creatine, is quite low in both cell types (<20%), a ratio which is lower than that in other tissues\textsuperscript{328-330} but which is in close agreement with published data concerning the extent of testicular creatine phosphorylation.\textsuperscript{249, 250, 252} The underlying cause of this relatively low extent of creatine phosphorylation is uncertain, but there are a number of possible reasons - the need for the maintenance of intratesticular concentration gradients, to allow diffusion of creatine between cells; a novel role for creatine which involves limited phosphorylation; cellular cross-contamination; or loss of PCr during isolation or extraction procedures.

Sertoli cells synthesise and, \textit{in vitro}, secrete creatine (Figures 4.3, 4.4A & 4.5, Table 4.3). This secretion may occur \textit{in vivo} to supply the germ cells, which contain creatine but do not synthesise it (Figures 4.3 & 4.4B, Table 4.1). Creatine secretion \textit{in vivo} could occur by active and/or passive mechanisms. Passive diffusion would require the maintenance of a high concentration gradient across the Sertoli cell plasma membrane.\textsuperscript{298 - 304}
Therefore it would be necessary for a high proportion of Sertoli cell creatine to be unphosphorylated.

The low extent of creatine phosphorylation in germ cells may reflect either a novel function for creatine, not requiring phosphorylation; or different extents of phosphorylation within the heterogeneous cell population. However, although human erythrocytes have been shown to sequester creatine by a two component system (i.e. active and passive mechanisms), and rat erythrocytes contain substantial creatine reserves, these cells do not express CPT activity.\textsuperscript{302, 304, 385} Human monocytes also sequester creatine, without expressing CPT activity (although differentiation of these cells into macrophages is associated with expression of CPT activity and the development of a PCr pool).\textsuperscript{302, 348} If there is any role for creatine within these cells it presumably involves non- or limited-phosphorylation pathways.

Further examination of creatine within isolated cells of the seminiferous epithelium will be required to determine the significance of the low extent of creatine phosphorylation, and to elucidate the role of creatine in these cells.

4.4.2 Testicular creatine synthesis.

4.4.2a Creatine is synthesised within the seminiferous epithelium.

The testis has the capacity to synthesise creatine, but previous investigations have used only whole tissue to examine this function.\textsuperscript{251, 331} Because creatine is associated with the seminiferous epithelium and not with the interstitial tissue (Chapter 3), then it was considered probable that this is also the site for creatine synthesis. Initial investigations into the synthesis of [\textsuperscript{14}C]creatine from L-[\textsuperscript{guanidino-14}C]arginine by isolated seminiferous tubules and a crude interstitial preparation showed that this assumption was correct. Whilst seminiferous tubules substantially converted L-[\textsuperscript{guanidino-14}C]arginine to both [\textsuperscript{14}C]GAA and [\textsuperscript{14}C]creatine (Figure 4.2, Table 4.2), the interstitial tissue had little synthetic activity (Table 4.2) and even this may have been due to contaminating tubular tissue.
It is interesting to note that the incorporation of radioactivity into GAA, relative to creatine, was very much lower in tubules isolated from immature rats, when compared to those from mature animals (Figure 4.2, Table 4.2). This may represent a difference in enzyme activities between the immature and mature testis, as a result of an age-related alteration in either the level of enzyme protein, or in the activity of enzymes already present. Alterations in enzyme activities within the testis as a whole have been reported to occur as the germ cell complement increases.\(^{387\,\,\,395}\) However, GAT and GMT are both associated with the Sertoli cell (Figures 4.4 & 4.5) and so any alteration in the level of enzyme protein, arising as a result of the change in the testicular cell population with age, may be expected to be reflected equally in the activity of both enzymes. Because these two enzymes are metabolically interdependent, it is unlikely that either would be expressed independently of the other. Therefore, the relative activities of the two enzymes in relation to each other probably remains unchanged.

Alternatively the difference could represent an alteration in the pool sizes of GAA and creatine with age. In consideration of this final point, it is important to note that the extent of GAA and creatine synthesis should not be confused with the incorporation of radioactivity, from arginine, into these products by isolated tubules. Because the substrate and product pool sizes in the tissues are unknown, the specific activities of the pools, and thus the extent of their metabolism, cannot be determined from radioactive tracer studies alone. Furthermore, there may be variations in the uptake of creatine into the testis from the plasma with age, and this will differentially affect the GAA and creatine pool sizes within the organ.

### 4.4.2b The cellular distribution of the enzymes involved in creatine synthesis within the seminiferous epithelium.

Because creatine synthesis could now be attributed to the seminiferous epithelium, further experiments with isolated and cultured cells were carried out to determine whether
synthesis was isolated within one cell type, or distributed throughout the epithelium. If the
total level of creatine in a cell culture (i.e. cells and medium together) is measured
immediately and 24 h after the replacement of medium, then any increase in the level of
creatine after 24 h is a measure of creatine synthesis by the cells (as opposed to leakage
into the medium, alterations in cellular compartmentation etc.). The total creatine content of
both SCEC and SGCC was determined in this manner (Figure 4.3). The total creatine
content of the SCEC increased by an average of 35 nmol over 24 h. Although the creatine
content of the SGCC at the start of the incubation (0 h) was more than double that of the
SCEC, a reflection of the creatine content of the germ cells, the increase over 24 h was not
much greater (43 nmol). From this it was deduced that creatine synthesis is carried out
chiefly by the Sertoli cells and not by germ cells.

This conclusion was confirmed by experiments in which SCEC and isolated germ
cells were incubated in the presence of \( L\)-[guanidino-\(^{14}\)C]arginine over 24 h. Sertoli cells
metabolised arginine, and incorporated the labelled amidine group into both creatine and
GAA. Germ cells did not exhibit this activity (Figure 4.4), although they may have the
capacity to synthesise creatine from GAA.

Sertoli cells also incorporated \([1-^{14}\)C]glycine into both creatine and GAA (Figure 4.5).
This is an interesting finding in consideration of the published data of Alekseeva and
Arkhangel'skaya.\(^{331}\) These workers demonstrated the synthesis of creatine, but not GAA,
from \([^{14}\)C]glycine by testicular tissue in vitro, although GAA synthesis by kidney tissue was
described, and they proposed a novel pathway for creatine synthesis within the testis to
explain this. Koszalka reported the synthesis of both GAA and creatine from \([^{14}\)C]arginine,
and of creatine from \([^{14}\)C]GAA by the testes of anaesthetised rats,\(^{261}\) indicating that testicular
tissue can synthesise both derivatives, and that creatine synthesis proceeds via the
production of GAA. The results presented in this Chapter clearly show that GAA and
creatine are produced from both arginine and glycine, and that creatine synthesis within the
testis follows the classical route; that is, transamidination between arginine and glycine,
followed by the methylation of the resultant GAA to creatine.\textsuperscript{253}

The greater incorporation of L-[guanidino-\textsuperscript{14}C]arginine than [\textsuperscript{1-14}C]glycine into GAA and creatine may be explained since glycine is a non-essential amino acid whereas arginine is essential in cases of limited or absent urea cycle activity, and glycine may also enter many more metabolic pathways than arginine. Thus the activity of exogenous radiolabelled glycine incorporated into creatine and GAA may be extensively reduced by dilution with 'cold' glycine synthesised by the cells, and by incorporation into other diverse cellular pathways.

\textbf{4.4.2c The significance of creatine synthesis within the testis.}

The skeletal muscle, heart, and brain also contain substantial creatine pools (Table 1.4) but do not carry out the two-stage synthesis of creatine themselves,\textsuperscript{331} although they appear to have the capacity to synthesise creatine from GAA.\textsuperscript{396} Active uptake of plasma creatine has been demonstrated for skeletal muscle,\textsuperscript{300, 301} and such an uptake system may also exist in the testis. The question that then arises is if the skeletal muscle, heart, and brain supplement their creatine pools solely from the blood, why does the testis have synthetic capacity, and not rely entirely upon uptake from the bloodstream?

The answer may lie in the fact that the testis has a relatively poor blood supply, and that skeletal muscle alone represents a substantial, and well supplied, 'creatine sink'. In addition, Free et al have suggested that some of the testicular blood flow may pass through non-metabolic routes, bypassing the testicular parenchyma.\textsuperscript{397} Thus, the full potential of the blood for supplementing the testicular creatine pool may not be realised. Under normal conditions, the availability of plasma creatine for uptake into the testis may be too low for its requirements.

Hyperthyroidism has been reported to increase the levels of creatine in the blood and urine, resulting in a reduction in creatine uptake by, and an increase in creatine efflux from, skeletal and cardiac muscle.\textsuperscript{370, 383} However, the reduction in the creatine content of skeletal and cardiac muscle following induced hyperthyroidism in rats is contrasted by an
increase in the testicular content. This may be as a direct result of T3 action upon the testis, since T3 receptors have been detected in cultured Sertoli cells, and the hormone has been shown to alter Sertoli cell function *in vitro*, or, alternatively, it could be due to an increase in sequestration by the tissue from the bloodstream, allowed by the elevated creatine concentration in the plasma.

Until the extent of creatine sequestration by the testis is known, it is difficult to determine whether synthesis acts as a supplement to or replacement for uptake from the plasma. However, it would appear reasonable to assume that for the testis to invest in the ability to synthesise a metabolite that other organs obtain from external sources, suggests that uptake from the plasma does not fulfill the testes' requirements for that metabolite.

### 4.4.3 Further studies.

The measurement of GAT and GMT activities in cultured Sertoli cells, and the effect of modulators of Sertoli cell function upon these activities, may aid further progress in the study of the relationship between creatine secretion and nitrogen metabolism in these cells (Chapter 5). The repression of GAT activity by creatine is thought to represent the key point of regulation for hepatic creatine synthesis, and it would be interesting to examine this in the Sertoli cell.

Many cell types that contain creatine cannot synthesise it from basic precursors, but have the ability to methylate GAA. Since cultured Sertoli cells secrete GAA as well as creatine, it will be of interest to determine whether or not germ cells are capable of synthesising creatine when incubated in the presence of [14C]GAA.
CHAPTER 5

THE SECRETION OF CREATINE BY CULTURED SERTOLI CELLS AND THE EFFECT OF MODULATORS OF CELLULAR BIOCHEMICAL FUNCTION
ABSTRACT.

Cultured Sertoli cells, maintained in a defined medium, secreted creatine into the incubation medium, in a manner which was linear with time over at least 6 h, but which reached a plateau within 24 h. The secretion of creatine was stimulated both by physiological (FSH, dbcAMP) and toxicological (MEHP, cadmium) modulators of Sertoli cell function. Stimulation of creatine secretion by FSH and dbcAMP was enhanced by the addition of an inhibitor of cAMP PDE activity to the incubation medium.

The secretion of creatine by SCEC, incubated over 4 h in EBSS, was independent of exogenous L-glutamine. However, the stimulation of creatine secretion induced by 1 mM dbcAMP was dependent upon the presence of 4 mM L-glutamine in the incubation medium. These observations suggest that an increase in creatine secretion may occur as a consequence of stimulated glutamine oxidation.
5.1 INTRODUCTION.

Cultured Sertoli cells synthesise a wide range of products, and secrete many of them into the incubation medium, including lactate, pyruvate, 4-methyl-2-oxopentanoate, ketone bodies, inositol, steroids, and proteins. Such secretory functions can be modified both by physiological regulators of Sertoli cell function and by Sertoli cell toxicants.

Hormones stimulate many changes in Sertoli cell character and function (Table 1.2). The effects of FSH are mediated via a cell-surface receptor which stimulates the activity of adenylate cyclase, resulting in an increase in the intracellular concentration of cAMP. The effects of FSH can be mimicked by dbcAMP, a lipid-soluble derivative of cAMP, and enhanced by the inhibition of cAMP catabolism (§1.2.2ai). FSH-induced modulation of Sertoli cell secretory functions have been suggested as a possible mechanism by which this hormone regulates spermatogenesis.

Modification of Sertoli cell functions have also been reported for a wide range of testicular toxicants, both in vivo and in vitro (Table 5.1). The most widely reported, and consistent, functional alteration is an increase in the secretion of lactate, induced by a broad spectrum of toxicants, including phthalate esters, 1,3-DNB, DNT isomers, cadmium, lead, 2,5-hexanedione and acrylamide.

Creatine is synthesised and secreted by SCEC (Chapter 4). The purpose of the experiments reported in this Chapter was to examine whether or not altered cellular homeostasis could play a role in regulating creatine metabolism by cultured Sertoli cells. Lactate secretion was measured as a positive control because it is altered by a wide range of Sertoli cell modulators.
Table 5.1. The effect of testicular toxicants on rat Sertoli cell function in vitro. This table is an illustrative summary of experiments into the effects of toxicants upon Sertoli cell function. Abbreviations: ↑, increased; ↓, decreased; NE, no effect.

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Functional Parameter</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAA</td>
<td>Glucose oxidation</td>
<td>↑</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Methoxyglucose sequestration</td>
<td>↑</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Lactate oxidation</td>
<td>↑</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Lactate secretion</td>
<td>↓</td>
<td>55, 56</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial membrane polarisation</td>
<td>↑</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Protein synthesis</td>
<td>NE</td>
<td>55</td>
</tr>
<tr>
<td>MEHP</td>
<td>Cell morphology</td>
<td>Altered</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Glucose oxidation</td>
<td>↑</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Pyruvate oxidation</td>
<td>NE</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Acetate oxidation</td>
<td>NE</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Lactate secretion</td>
<td>↑</td>
<td>56, 77, 78</td>
</tr>
<tr>
<td></td>
<td>Pyruvate secretion</td>
<td>↓</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Lipid synthesis</td>
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<tr>
<td></td>
<td>SDH activity</td>
<td>↓</td>
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<td>Intracellular ATP levels</td>
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<td>78</td>
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<td></td>
<td>FSH-induced stimulation of adenylate cyclase activity</td>
<td>↓</td>
<td>80-82</td>
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<tr>
<td></td>
<td>Transferrin secretion</td>
<td>↓</td>
<td>403</td>
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<tr>
<td>1,3-DNB</td>
<td>Lactate secretion</td>
<td>↑</td>
<td>56, 92</td>
</tr>
<tr>
<td></td>
<td>Pyruvate secretion</td>
<td>↑</td>
<td>56, 92</td>
</tr>
<tr>
<td>DNT isomers</td>
<td>Cell morphology</td>
<td>Altered</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Lactate secretion</td>
<td>↑</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Pyruvate secretion</td>
<td>↑</td>
<td>92</td>
</tr>
<tr>
<td>Cadmium</td>
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<td>2,5-Hexanedione</td>
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<td>Thio-D-glucose</td>
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Figure 5.1. The structures of Sertoli cell modulators used in these studies. (A) DbcAMP (sodium salt), (B) MEHP. The structure of FSH, a large (approximately 36 kD) glycoprotein consisting of two sub-units linked by disulphide bridges, is not given.
5.2 METHODS.

5.2.1 Pretreatment of SCEC.

SCEC were cultured for a total of 72 h after isolation, prior to commencement of all studies, i.e. studies were undertaken 24 h after germ cell removal.

5.2.2 Preparation of test solutions.

Water-soluble test compounds were dissolved directly in the culture medium, except cadmium, which was made up as a 1000-fold stock solution in deionised water and diluted appropriately with the culture medium.

Hydrophobic test compounds (testosterone, MEHP) were made up as 400-fold stock solutions in DMSO and diluted appropriately with the culture medium. Control cultures were exposed to 0.25% DMSO.

The FSH used was a commercial preparation derived from porcine pituitary (pFSH, Sigma catalogue Nº F8001, Lot Nº 88F 0226). It had an activity of 2.04 U/mg solid, unit definition as given by Steelman and Pohley.407

5.2.3 Sample collection and storage.

Media were removed from the cultures and centrifuged (45 g, 5 min) to remove cells and debris. The supernatants were stored at -80°C prior to biochemical assays (§2.8.1 & §2.8.2).

Culture dishes were stored at -20°C prior to determination of protein (§2.7.4). The cell monolayer was digested overnight at room temperature in sodium hydroxide (1 M) and assayed for protein content, which served as an indicator of cell plating density.
5.3 RESULTS.

5.3.1 The secretion of creatine and lactate by SCEC under basal conditions.

Creatine and lactate secretion by Sertoli cells cultured in supplemented EMEM for 24 h was compared for cells that were cultured at different densities. Secretion did not show any obvious correlation with the cellular protein content of the dishes (Figure 5.2).

The secretion of creatine and lactate by confluent monolayers of Sertoli cells did increase with the size of the culture dish. Cultures in 50 mm diameter dishes (plating area = 2500 mm²) secreted approximately twice as much creatine and lactate as those in 35 mm diameter dishes (plating area = 1225 mm²).

Therefore, the secretion data presented in this Chapter has not been related to cellular protein but is expressed as the total secretion by the cultured cells (i.e. as nmol/dish and μmol/dish for creatine and lactate respectively).

![Figure 5.2](image_url)

**Figure 5.2. The secretion of creatine and lactate by Sertoli cells cultured at different cell densities.** SCEC were cultured in 50 mm (closed symbols) or 35 mm (open symbols) diameter dishes, in supplemented EMEM, for 24 h. The media were removed and assayed for (A) creatine and (B) lactate. The cellular protein content of the cultures was determined after digestion overnight in 1 M sodium hydroxide. The data represent the results for duplicate determinations on 4-6 dishes from different cell preparations.
SCEC secreted creatine linearly with time, at a mean rate of 6.6 nmol/dish/h, over the first 6 h of incubation (Figure 5.3). Secretion had reached a plateau by 24 h, by which time the extracellular creatine concentration had reached 11.0-15.3 μM. The range of concentrations after 24 h incubation in the absence of exogenous stimuli for all SCEC preparations used in this course of studies was 10-30 μM.

The secretion of lactate followed a similar pattern. Secretion occurred at a mean rate of 0.41 μmol/dish/h, reaching a plateau at 6.5-7.7 mM after 24 h incubation.

Figure 5.3. The time course for the secretion of creatine and lactate by SCEC. SCEC were maintained on 50 mm plastic petri dishes in supplemented EMEM for 2, 4, 6 or 24 h after which time the medium was removed and assayed for creatine and lactate. The medium from randomly selected dishes was removed and replaced at t = 0 h. The data are the mean ± SD for secretion values from two (lactate, closed circles) or three (creatine, open circles) cell preparations, each value being the mean of duplicate determinations from 6 dishes.
5.3.2 The effect of modulating intracellular cAMP upon creatine secretion by SCEC.

The addition of pFSH to the culture medium resulted in a stimulation of creatine secretion by SCEC which was statistically significant at higher concentrations when data from each cell preparation were considered individually (not shown), but which was not significant when the results from all three preparations were combined (Figure 5.4).

The concentration of FSH required to significantly stimulate lactate secretion above control levels (1.96 μg/ml) was much higher than that required by more highly purified preparations of the hormone (e.g. 5 ng/ml for NIH-FSH-S13), but is in broad agreement with published data obtained with this commercial preparation. Other workers have shown...

Figure 5.4. The effect of FSH upon creatine and lactate secretion by SCEC. The secretion of creatine (open circles) and lactate (closed circles) by cultured Sertoli cells after exposure to various concentrations of pFSH (Sigma pFSH-88F 0226) in supplemented EMEM for 24 h. The data are the mean ± SEM for measurements from 3 preparations, duplicate determinations were made for 6 dishes. * Significantly different from control (Student's two-tailed t-test, P <0.05).
that the concentration of FSH required to elicit a given response is reduced as the purity of
the hormone preparation increases.\textsuperscript{169}

The addition of dbcAMP to the incubation medium also stimulated creatine and
lactate secretion by Sertoli cells. The threshold concentration for this effect was 0.1 mM
(Figure 5.5).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5_5.png}
\caption{The effect of dbcAMP upon creatine and lactate secretion by SCEC. The secretion of
creatine (open circles) and lactate (closed circles) by cultured Sertoli cells after exposure to various
concentrations of dbcAMP in supplemented EMEM for 24 h. The data are the mean ± SEM for
measurements from 3 preparations, duplicate determinations were made for 6 dishes. * Significantly
different from control (Student’s two-tailed t-test, \(P < 0.05\)).}
\end{figure}
In one experiment the stimulation of creatine secretion by both pFSH and dbcAMP was enhanced by the addition of 3-isobutyl-1-methylxanthine (MIX), a cAMP PDE inhibitor, to the culture medium (Figure 5.6).

Creatine secretion by SCEC exposed to pFSH alone was stimulated by 71% with respect to control, but this was increased to 171% in the presence of 0.5 mM MIX. The dbcAMP-induced stimulation of creatine secretion by SCEC was increased from 109% in the absence of MIX to 237% in the presence of 0.5 mM MIX.

MIX had little effect upon creatine secretion when added to cultures on its own, increasing secretion on average by 17%.

![Figure 5.6](image-url)

**Figure 5.6.** The effect of PDE inhibition upon FSH- and dbcAMP-induced stimulation of creatine secretion. Cultures were incubated in the presence of pFSH (0.4 U/ml) or dbcAMP (1 mM) in supplemented EMEM, with or without MIX (0.5 mM), for 24 h. The medium was removed, and its creatine content determined. The data are the results of duplicate determinations from groups of 6 culture dishes.
5.3.3 The effect of L-glutamine upon creatine secretion.

Under basal conditions SCEC secreted creatine in a manner which was independent of exogenous glutamine (6.1 and 6.5 nmol/dish/4 h in the absence and presence of 4 mM L-glutamine respectively, Figure 5.7). The secretion of creatine was stimulated by more than 75% by dbcAMP in the presence of glutamine, but this stimulation was reduced to only 15% when the amino acid was omitted from the medium (7.0 and 11.5 nmol/dish/4 h in the absence and presence of 4 mM L-glutamine respectively).

The addition of L-glutamine to the incubation medium did not affect the production of lactate by SCEC, nor the dbcAMP-induced stimulation of lactate secretion (Figure 5.7).

Figure 5.7. The effect of exogenous L-glutamine upon creatine secretion by SCEC. SCEC were incubated in EBSS, with or without L-glutamine (4 mM) and dbcAMP (1 mM), for 4 h. The medium was then removed, and assayed for (A) creatine and (B) lactate. The data presented are the mean and range for two separate cell preparations, each being the mean of duplicate determinations from 4 or 6 dishes.
5.3.4 The effect of Sertoli cell toxicants upon the secretion of creatine by SCEC.

MEHP stimulated the secretion of creatine by SCEC, in an apparently concentration-dependent manner, over 24 h. Lactate secretion was also stimulated in a similar fashion (Figure 5.8). The threshold concentration for the effect upon both creatine and lactate secretion appears to be the same (1.0 μM).

MEHP did not affect cell viability as assessed by trypan blue exclusion. The viability of cultures exposed to the highest concentration of MEHP (100 μM) was 98%, compared to 99% for control cultures.

![Figure 5.8. The effect of MEHP upon creatine and lactate secretion by SCEC.](image)

The secretion of creatine (open circles) and lactate (closed circles) by cultured Sertoli cells after exposure to various concentrations of MEHP in supplemented EMEM for 24 h. The data are the mean ± SEM for measurements from 4 (a), 3 (b) or 2 (c) preparations, duplicate determinations were made for 6 dishes. * Significantly different from control (Student's two-tailed t-test, $P<0.05$).
Cadmium stimulated the secretion of lactate by SCEC at concentrations as low as 0.3 μM, whereas creatine secretion was stimulated only at concentrations of 1 μM and above (Figure 5.9). Cadmium did not affect cell viability at 1 μM, as assessed by trypan blue exclusion (97%, cf. 99% for control cultures), but reduced viability to 93% and 68% at 3 μM and 10 μM respectively.

**Figure 5.9.** The effect of cadmium upon creatine and lactate secretion by SCEC. The secretion of creatine (open circles) and lactate (closed circles) by cultured Sertoli cells after exposure to various concentrations of cadmium in supplemented EMEM for 24 h. The data are the mean ± SEM for measurements from 3 (creatine) or 2 (lactate) preparations, duplicate determinations were made for 6 dishes. * Significantly different from control (Student's two-tailed t-test, P <0.05).
5.4 DISCUSSION.

The cultures used in these studies were confluent monolayers of Sertoli cells with little germ cell contamination (SCEC, Figure 2.4). The identity of the cells was confirmed by morphological and biochemical responses to FSH (assumption of a spindle-like shape, stimulation of lactate secretion). The response of the SCEC to modulators used in these studies was in good agreement with published data.

The viability of the cultured cells was estimated by trypan blue exclusion and found to be >99%.

5.4.1 The synthesis and secretion of creatine by cultured Sertoli cells.

In Chapter 4, cultured Sertoli cells were shown to synthesise creatine, incorporating the radioactivity from both \[^1^4\text{C}]\text{glycine and } L-[\text{guanidino-}^1^4\text{C}]\text{arginine. }[^1^4\text{C}]\text{Creatine produced by the cells was released into the incubation medium by as yet unknown mechanisms. These may be passive and/or active processes, but for the purposes of discussion the term 'secretion' will be used.}

SCEC cultured in a medium containing both glycine and L-arginine secreted creatine at a steady rate of 6.3 nmol/mg protein/h for at least 6 h but reached a plateau by 24 h, at which time the concentration of creatine in the medium was 10-30 \text{\textmu M (Figure 5.3). This indicates that creatine synthesis is linear with respect to time, until the intracellular and/or extracellular concentration reaches a threshold level, when feedback inhibition of synthesis probably occurs (Chapter 4). The rate of secretion was slightly reduced to 4.1 nmol/mg protein/h when cells were incubated in EBSS (Figure 5.7). It is not clear whether or not this represents an impairment of creatine synthesis in the absence of exogenous precursors. Under such conditions secreted creatine may be derived from endogenous stores (e.g. dephosphorylation of PCr).}

The apparent independence of creatine secretion from cell plating density is a phenomenon that has previously been noted for the secretion of lactate by cultured Sertoli
cells. It is unclear as to what are the precise underlying mechanisms, but if secretion is controlled by negative feedback pathways related to the concentration of creatine in the extracellular milieu (in this case the incubation medium), then such feedback will be the overriding factor in determining the kinetics of secretion, and cell density will have less of an effect. This is a fundamental difference in monitoring the levels of intracellular and extracellular metabolites, the former are related directly to the number of cells present, but this is not necessarily the case for the latter.

Cultured Sertoli cells extensively oxidise exogenous amino acids such as L-glutamine and L-leucine (6.8 and 13.0 nmol/mg protein/h respectively). Both stages in the oxidation of glutamine to 2-oxoglutarate liberate ammonia, and the complete oxidation of glutamine by SCEC will produce approximately 14 nmol ammonia/mg protein/h. If Sertoli cells have the capacity to detoxify ammonia by incorporating nitrogen into arginine, then the oxidation of glutamine could be expected to be an important stimulus for the secretion of creatine. However, this was not found to be the case. When SCEC were incubated in EBSS (a simple balanced salt solution containing D-glucose) over 4 h, the secretion of creatine was not altered by the addition of 4 mM L-glutamine to the medium (Figure 5.7). It appears that exogenous amino acids are not essential for the maintenance of creatine secretion under basal conditions.

5.4.1a The influence of cAMP.

Creatine secretion by SCEC was stimulated by the presence of FSH and dbcAMP in the incubation medium (Figures 5.4 and 5.5). This stimulation was further enhanced by the MIX-induced inhibition of PDE activity (Figure 5.6), confirming that it results from an elevation in intracellular cAMP levels.

FSH stimulates the sequestration of 3-O-methyl-D-glucose by Sertoli cells, and the

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* T.J.B. Gray. *Personal communication.*
glucose flux through many pathways (e.g. glycolysis, pentose phosphate pathway, TCA cycle, inositol biosynthesis) is stimulated by both FSH and dbcAMP.\textsuperscript{148, 198, 401} The secretion of lactate by cultured Sertoli cells, and its stimulation by FSH, are dependent upon the concentration of glucose in the incubation medium.\textsuperscript{152, 197}

The oxidation of \([U-^{14}C]\)glutamine by cultured Sertoli cells is stimulated by 69\% in the presence of 0.5 mM dbcAMP (from 6.8 to 11.5 nmol/mg protein/h).\textsuperscript{148} Over a 24 h incubation period dbcAMP stimulated creatine secretion by SCEC, maintained in a defined medium supplemented with 4 mM L-glutamine, by 55\% at a concentration of 0.1 mM, and by 96\% at 1.0 mM (Figure 5.5). In experiments carried out over a 4 h time course, with an incubation medium consisting of EBSS supplemented with 4 mM L-glutamine, 1.0 mM dbcAMP stimulated creatine secretion by 75\%. Stimulation was reduced to only 15\% when L-glutamine was omitted from the medium (Figure 5.7). Therefore, although the basal secretion of creatine was apparently independent of exogenous L-glutamine, the presence of the amino acid in the incubation medium was essential for the stimulation of secretion induced by dbcAMP. The stimulation of creatine secretion induced by dbcAMP (and FSH) is probably due, at least in part, to the increased oxidation of L-glutamine.

Under basal conditions, Sertoli cells derive much of their energy from lipids.\textsuperscript{402} Review of the literature did not reveal any published data concerning the effect of cAMP upon palmitate oxidation. However, FSH has a stimulatory effect upon fatty acid biosynthesis,\textsuperscript{185} and the oxidation of leucine, which enters energy-yielding pathways in a similar manner to lipids (i.e. as acetyl CoA), is not stimulated by dbcAMP,\textsuperscript{148} so it appears that cAMP would not increase palmitate oxidation. On the other hand, both glucose oxidation through the TCA cycle and glutamine oxidation are stimulated by dbcAMP.\textsuperscript{148} Therefore, under conditions of increased energy utilisation the additional energy may come from alternative sources such as glucose and certain amino acids.

The mechanism by which cAMP stimulates glutamine oxidation is unclear,\textsuperscript{148} but it may be due to a direct effect upon the mitochondrion or to an alteration in the energy charge.
of the cell. ATP, GTP and NADH are allosteric inhibitors of glutamate dehydrogenase, whereas ADP and GDP are allosteric activators. A reduction in the ATP/ADP ratio within the cell will increase the activity of this enzyme, thereby increasing the oxidation of glutamate to 2-oxoglutarate. The glutamate required by the enzyme can be derived from four amino acid precursors; arginine, glutamine, histidine, and proline. Hence oxidation of these amino acids may be stimulated in order to supply the energy required for other alterations in Sertoli cell activity induced by cAMP. The stimulation of amino acid oxidation will result in an increase in the production of ammonia, which may be detoxified by incorporation into urea or creatine. There is at present no evidence that Sertoli cells undergo ureagenesis. The alternative pathway for detoxification of ammonia is by amination of glutarate to glutamine, but clearly this is not logical under conditions of increased glutamate/glutamine oxidation.

Lactate secretion is altered by a direct effect upon its immediate precursor, glucose. As glucose uptake is stimulated, so too is the flux through the glycolytic pathway, and thus the production of lactate from pyruvate. Creatine secretion appears to be altered by a more indirect effect, i.e. an energy imbalance caused by an increase in energy consumption, resulting in a stimulation of amino acid oxidation and, consequently, ammonia production. This more indirect mechanism for affecting creatine synthesis would mean that it will be less sensitive to alteration, and so will only be stimulated at higher concentrations and/or over longer exposure periods.

It must be borne in mind that arginine interferes with the determination of creatine in EMEM, and measurements must be adjusted accordingly. This may introduce errors which could mask small but significant differences. For example, 0.4 U/ml FSH had no effect upon creatine secretion by SCEC into EMEM during time periods of less than 24 h (data not shown), but 1 mM dbcAMP stimulated secretion into EBSS within 4 h (Figure 5.7), although at these concentrations both stimulated creatine secretion into EMEM to a similar extent over 24 h. This may reflect the slightly different modes of action of dbcAMP and FSH upon the Sertoli cell, but may alternatively result from the greater accuracy of creatine determination in
EBSS (after 4 h incubation, the percentage error for the measurement of creatine was 6% and 20% for EBSS and EMEM respectively).

5.4.1b The influence of toxicants.

i) MEHP. MEHP stimulated the secretion of creatine in an apparently concentration-dependent manner (Figure 5.8). This was not due to increased leakage of creatine from cells since MEHP does not cause significant cell death nor does it increase plasma membrane permeability to ATP, ADP and trypan blue at the highest concentration employed in these studies (100 μM). \(^{77, 78}\)

After 3 h exposure of Sertoli cells to 0.1 mM MEHP, glucose utilisation and oxidation within the TCA cycle are stimulated by 67% and 21% respectively. \(^{76}\) However, although the potential ATP production from glucose metabolism is increased, from 9.7 to 14.0 nmol/mg prot/min (estimated from the data of Worrell et al.\(^{76}\)), the actual ATP content of the cells is not altered at this time, and is decreased to 85% of control by 24 h. \(^{78}\) Therefore, MEHP seems to affect the Sertoli cell in such a manner as to increase its energy consumption (e.g. increased lipid synthesis and cytoskeletal changes). \(^{74, 76, 78}\)

Sertoli cells contain creatine in both the free and phosphorylated states, and express CPT activity (Chapter 4). If the energy charge of the cell was to fall as a result of exposure to MEHP, then ADP could be rephosphorylated at the expense of PCr. The result would be to increase the intracellular free creatine concentration and could result in an increase in the movement of creatine out of the cells, with a concomitant decrease in cellular levels, since biological membranes are more permeable to creatine than to PCr, \(^{408}\) allowing creatine to more readily cross the plasma membrane by passive pathways. However, the level of PCr measured in these cells (16%, Table 4.1) would not be enough to account for the extent of stimulation (135% at 100 μM MEHP), although the actual PCr concentration within the Sertoli cells may of course be higher.

There are no data concerning the effects, if any, of MEHP upon amino acid
metabolism in cultured Sertoli cells. Although the phthalate esters have been reported to inhibit succinate dehydrogenase activity, MEHP does not inhibit the oxidation of exogenous acetate and pyruvate and actually stimulates glucose oxidation by the TCA cycle. This suggests that the activity of the TCA cycle is not compromised by MEHP in situ and that cells retain the capacity to oxidise the carbon skeletons of amino acids. Therefore MEHP may stimulate creatine secretion in a similar manner to that proposed for dbcAMP (FSH), above.

**ii) Cadmium.** Cadmium stimulated lactate secretion at concentrations as low as 0.3 μM, whereas creatine secretion was not stimulated below 3 μM (Figure 5.9). Cadmium does not reduce Sertoli cell viability at concentrations up to 1 μM, but viability was substantially reduced at 10 μM. The threshold cadmium concentration for both stimulation of creatine secretion and reduction of cell viability appears to be around 3 μM. Therefore, an increase in leakage of creatine from cellular stores must be considered as an important contributing factor in the elevation of extracellular creatine levels after exposure to higher concentrations of cadmium.

Clough et al reported that cadmium reduces glucose oxidation at concentrations as low as 0.25 μM and causes a dose-related increase in lactate secretion, suggesting that at low concentrations it interferes with the balance of energy production in the cell. Since cadmium reduces glucose oxidation by Sertoli cells, the activity of the TCA cycle is probably reduced and therefore stimulations in amino acid oxidation and creatine synthesis are unlikely.

There are no reports in the literature on the effect of cadmium treatment upon ATP levels in Sertoli cells. Reduction of the energy charge of the cell may increase intracellular free creatine levels by stimulating the dephosphorylation of PCr, but as for MEHP the measured PCr content of these cells is not enough to account for the stimulation in creatine secretion (66% at 10 μM cadmium).
5.4.2 Conclusions.

In the experiments reported in this Chapter, a range of Sertoli cell functional modulators have been shown to increase the secretion of creatine by SCEC. All of these compounds alter energy metabolism by Sertoli cells, and their effects may be mediated by a depression of the energy charge of the cell, which would result in increased creatine synthesis, as amino acid oxidation and ammonia production are stimulated, and / or increased dephosphorylation of PCr. Either of these activities would elevate the free creatine content of the cells, and thus stimulate creatine leakage / secretion.

In the dose-response studies, the secretion of creatine was less sensitive to stimulation than that of lactate. This was particularly marked in the case of cadmium intoxication, in which stimulation of the medium creatine levels was only consistently observed at concentrations which were overtly cytotoxic (i.e. 3 \( \mu \)M and 10 \( \mu \)M). Therefore, it appears that the alterations in nitrogen metabolism induced by these modulators are probably a more indirect effect than the observed changes in carbon metabolism, occurring further down the cascade of induced alterations in cellular biochemistry, although further studies into the causes and effects of altered nitrogen metabolism are required before firm conclusions can be drawn.

However, alterations in nitrogen metabolism may be one result of the effects of hormones and toxicants upon the Sertoli cell. This should come as no surprise, yet the possible role of altered nitrogen metabolism in the course of hormonal stimulation or toxic stress is rarely, if ever, examined. Many studies are made into carbon (particularly glucose) and phosphorus (ATP, ADP) metabolism, yet fewer considerations are made to other, vital areas of cellular biochemistry. The role of nitrogen metabolism in the course of hormonal or toxic action may only be peripheral, but until further examinations are made, this will remain speculation. The secretion of creatine may offer a convenient index for nitrogen metabolism in cultured Sertoli cells, but this will require validation.

One important point to come from these studies is that hormones and toxicants may
alter many more metabolic pathways, either directly or indirectly, than have up until now been considered in the study of their mechanisms of action. Until the full picture of biochemical alterations is built up, the research into these mechanisms, and their further implications for cellular homeostasis and interaction, may be hindered. The full understanding of cellular function, and changes in function, requires a full knowledge of the biochemistry of the cell, its control, and the interactions between related metabolic pathways.

5.4.3 Further studies.

Further understanding of the mechanisms underlying the changes in creatine secretion induced by modulators of Sertoli cell function may be gained from examining

a) cellular creatine, PCR, ATP, ADP, and AMP levels, and amino acid oxidation under different stimuli,

b) the effect of the presence and absence of exogenous amino acids upon creatine secretion,

c) the correlation of ammonia production with creatine synthesis under different conditions will enable the relationship between nitrogen metabolism and creatine secretion to be determined,

d) the incorporation of $^{15}$N from L-[amido-$^{15}$N]glutamine into creatine would allow the metabolic route for nitrogen metabolism to be determined.
CHAPTER 6

CREATINE UPTAKE BY ISOLATED GERM CELLS
ABSTRACT.

Freshly isolated preparations of germ cells sequestered [1-3H]creatine in a time-dependent manner, which was linear over at least 3 h.

Uptake was by a two component process. One component, which had a high affinity for creatine ($K_m = 25 \mu M$), and was inhibited both at low temperature and by the absence of sodium ions from the medium, probably represents an active uptake system. The other component, which had a low affinity for creatine (accounting for less than 30% of uptake at physiological creatine concentrations), was independent of temperature and sodium, and probably represents passive diffusion.

The affinity of the active uptake system for creatine was within the range reported for other known systems (20-60 $\mu M$), and also within the range of steady-state creatine concentrations found in the incubation medium above cultured Sertoli cells (10-30 $\mu M$). However, the uptake of creatine proceeded at a very low rate in comparison to other known systems ($V_{\text{max}} = 244 \text{ pmol/mg protein/3 h}$, cf. 700-5100 pmol/mg protein/h). The possible reasons for this are discussed.
6.1 INTRODUCTION.

Creatine has been located within both the Sertoli and germ cells of the seminiferous epithelium. Cultured Sertoli cells were shown to synthesise and release creatine, whereas the germ cells did not display synthetic activity (Chapters 4 & 5). This suggests that the germ cells may be supplied with creatine that is synthesised by, and released from, the Sertoli cells. For such an intercellular metabolic pathway to work efficiently, there must be an uptake system within the germ cell population to allow sequestration.

Creatine uptake has been demonstrated in a variety of tissues and cell types including skeletal muscle, smooth muscle, fibroblasts, macrophages, erythrocytes, and adipose tissue. The uptake of creatine is generally considered to be mediated by a two-component system, an active uptake component and a component of passive diffusion.

The aim of the experiments reported in this Chapter was to broadly define the uptake of creatine by a crude preparation of isolated germ cells.
6.2 METHODS.

All plastic ware was incubated overnight with a BSA solution (2 mg/ml, 1 ml), to reduce non-specific cell adhesion.

6.2.1 Linearity of uptake.

Germ cells were isolated, and cultured on 10 cm petri dishes, in supplemented EMEM, for various periods of time. At the end of this incubation, the cells were resuspended by agitation of the dishes, washed, and finally resuspended at a density of 1-2 x 10^6 cells/ml, in EBSS supplemented with 2 mM pyruvate and 6 mM L-lactate. Aliquots (0.5 ml) were added to 1 cm multiwells.

Aliquots (0.5 ml) of [1-^14C]creatine solution (approximately 1.0 µCi/ml in supplemented EBSS) were added to each well, to give a final creatine concentration of 30-50 µM. After specified times the cells were removed, washed twice with 10 mM creatine in 0.9% ice-cold saline, and finally suspended in ice-cold saline (1 ml). An aliquot (0.1 ml) was removed for cell counting. The remaining cells were pelleted and dissolved in NaOH (1 M, 0.45 ml). Each sample was neutralised by the addition of HCl (1 M, 0.45 ml), and 0.45 ml of this solution was then sampled for assessment of radioactivity.

6.2.2 Dependence of creatine uptake upon substrate concentration, temperature, and sodium ion concentration.

The incubation buffer used in these studies has been described (§2.11).

Freshly isolated cells were suspended (1-2 x 10^6 cells/ml) in the two incubation buffers (with or without 140 mM NaCl), and 0.5 ml aliquots were added to 1 cm multiwells containing [1-^14C]creatine (0.25 µCi/ml), and different concentrations (between 20 µM and

\[ T.J.B. Gray. Personal communication. \]
5 mM) of ‘cold’ creatine, in the respective buffers.

The cells were incubated at 32 °C or 4 °C for 3 h, after which time they were removed, washed three times with 10 mM creatine in 0.9% ice-cold saline, and dissolved in NaOH (1 M, 1 ml) overnight.

An aliquot (0.5 ml) of the digest was neutralised with HCl (1 M, 0.5 ml), and ‘Aquasol’ (4 ml) was added for the determination of radioactivity. A further 50 µl aliquot of the digest was taken for protein determination.

6.2.3 Determination of uptake kinetics.

To determine the kinetics of the active transport system, the diffusional component at each concentration of creatine was first subtracted from the measured uptake. The diffusional component for each concentration was calculated by extrapolating the uptake at saturation concentrations (i.e. 1.0 mM and 2.5 mM) to the origin, and this uptake was then subtracted from the total at each concentration, to determine the active component.

The values of \( K_m \) and \( V_{max} \) for the active uptake system were then calculated from Lineweaver-Burk plots of the data.
6.3 RESULTS.

6.3.1 Creatine uptake by cultured germ cells.

The uptake of creatine by cells cultured for different periods of time is shown in Figure 6.1. Uptake was greatest in cells that were freshly isolated and was linear for 3-4 h.

The loss of creatine transport capacity over the first 24 h of incubation (Figure 6.1B & 6.1C) was accompanied by an extensive loss of germ cells which were noted to adhere to a monolayer of Sertoli cells. In the first experiment (Figure 6.1A) there were few Sertoli cells and little germ cell loss.
Figure 6.1. The uptake of $[^{14}\text{C}]$creatine by isolated germ cells. The cells were preincubated for 1 h (closed circles), 24 h (closed squares), or 48 h (closed triangles) prior to the assessment of creatine uptake. The data represent the mean of three measurements from three separate cell preparations (A, B and C). The yield of germ cells from the cultures at 24 h and 48 h was drastically reduced in preparations B and C due to adhesion to a monolayer of Sertoli cells.
6.3.2 Dependence of creatine uptake upon the concentration of creatine in the extracellular medium.

Creatine uptake increased with the concentration of creatine in the incubation medium (Figure 6.2). This was not linear at low concentrations (Figure 6.3A), but appeared to become linear at higher concentrations. A Lineweaver-Burk plot of the data obtained at sub-saturation concentrations is shown in Figure 6.3B. The system for creatine transport had an apparent $K_m$ for creatine of 36 $\mu$M and the maximum rate of uptake ($V_{\text{max}}$) was 372 pmol/mg protein/3 h. The $K_m$ and $V_{\text{max}}$ for the active uptake system alone were 25 $\mu$M and 244 pmol/mg protein/3 h respectively.
Figure 6.2. Creatine uptake as a function of concentration. Germ cells were incubated in the presence of $[^{14}\text{C}]$ creatine, along with various concentrations of unlabelled creatine, in order to determine the effect of extracellular concentration upon the uptake of creatine into these cells.
Figure 6.3. The active uptake of creatine by isolated germ cells. (A) The component of passive diffusion (dashed line) was calculated by extrapolation and subtracted from all measurements of total uptake (solid line, closed circles) to derive the active component (solid line, open circles). The data represent the mean values from two cell preparations. (B) Lineweaver-Burk plots for the total (closed circles) and active (open circles) uptake of creatine. The apparent $K_m$ for the combined uptake was 36 μM, and the $V_{max}$ was 372 pmol/mg protein/3 h. The active uptake system had a $K_m$ for creatine of 25 μM, and a $V_{max}$ of 244 pmol/mg protein/3 h.
6.3.3 Dependence of creatine uptake upon temperature and the concentration of sodium ions in the incubation medium.

When freshly isolated germ cells were incubated in supplemented EBSS at 4°C, creatine uptake was substantially diminished, to the level of passive diffusion. The same effect was observed when cells were incubated in a sodium-deficient medium (sodium replaced with choline) at 32°C (Figure 6.4).

![Figure 6.4. The uptake of creatine by isolated germ cells incubated under different conditions of temperature and concentration of sodium ion in the incubation medium. Freshly isolated germ cells were incubated at 32°C in the presence of 142 mM Na⁺ (closed circles), at 4°C in the presence of 142 mM Na⁺ (closed squares), or at 32°C in the presence of 2 mM Na⁺ (closed triangles). The results presented are the mean ± SEM from two separate cell preparations, each representing the mean of duplicate determinations from 4 wells. * Significantly different from other treatment groups at this concentration of creatine. The dashed line represents the estimated component of passive diffusion.]
6.4 DISCUSSION.

6.4.1 The uptake of creatine by germ cells.

Germ cells contain substantial creatine stores, but do not have the capacity to synthesise creatine themselves (Chapter 4). Therefore, whether germ cell creatine is derived from Sertoli cell synthetic activity, or indirectly from the blood stream, there must be an uptake system to allow its entry at physiological concentrations.

Creatine uptake systems have been widely described, and extensively characterised. The characteristics of the uptake system described in this Chapter for the male germ cells are in close agreement with the published data for other cell and tissue types.

The active uptake system has a high affinity for creatine \( (K_m = 25 \, \mu M) \), which is in the range reported for other cells (20-60 \( \mu M \)). Interestingly, this value for the \( K_m \) is also within the range of steady-state creatine concentrations measured in the medium above cultured Sertoli cells (10-30 \( \mu M \), Chapter 5). Although the concentration of creatine secreted by Sertoli cells in vitro cannot be accepted as an accurate measure of that achieved in vivo (i.e. the extracellular creatine concentration within the seminiferous epithelium), it may serve as an indicator, since there appears to be a feedback product inhibition of creatine secretion (Chapter 5) which, presumably, is functional in vivo. If this is the case, then the \( K_m \) for germ cell creatine uptake reported in this Chapter may indeed be sufficient to allow efficient creatine sequestration in vivo. The importance of an active uptake system in these cells is obvious, since at physiological creatine concentrations passive processes played only a minor role in mediating creatine entry into the cells (<30% of the total).

When germ cells were incubated at low temperature or in sodium-depleted medium, there was no difference between the measured uptake of creatine and the calculated diffusional component of uptake, and uptake was essentially linear with respect to concentration (Figure 6.4). This suggests that under these conditions uptake was solely a passive process, and that the active transport of creatine into these cells was completely
inhibited. Therefore, active creatine transport is totally dependent upon both energy production, and the presence of extracellular sodium ions.

The data presented in this Chapter are derived from experiments carried out using freshly isolated germ cells, because of cell loss due to adhesion to contaminating Sertoli cells during culture (Figure 6.1). The isolation procedure involves the use of trypsin at a stage when the germ cells could be highly exposed to this proteolytic enzyme. The sequestration of creatine by human erythrocytes is inhibited by another proteolytic enzyme, pronase. Therefore, the (partial) removal of protein that may mediate germ cell creatine sequestration must be considered as a possible influential factor in the measurement of uptake parameters.

The value of $K_m$ may not be altered greatly by the isolation treatment, since it is a measure of the affinity of a protein for its substrate, and is an intrinsic property of that protein. If uptake sites remain active on the surface of isolated cells, then the $K_m$ can be measured with some confidence, if it is assumed that the effect upon activity of individual carrier protein molecules is 'all or nothing', and that diminution of activity of the carrier population as a whole is due solely to removal of protein molecules. Indeed, as previously stated, the affinity for creatine measured in germ cells is similar to values reported for other systems, and this can give some confidence to the biological validity of the calculated value for $K_m$.

However, $V_{max}$ is a property of the whole population of uptake sites on a cell surface. The greater the number of sites, the greater will be the maximal initial rate of uptake. Therefore, $V_{max}$ will be very much affected by cell isolation using proteolytic enzymes. The values for the rate of uptake reported in other cell systems are very much higher than that measured in these experiments. Reports for uptake by human and rat muscle cells and human macrophages lie in the range 2900-5100 pmol/mg protein/h. Even human monocytes, which don't utilise creatine or express CPT activity, sequester creatine at a rate 700 pmol/mg protein/h.298, 299, 302
After cells are isolated, they are often cultured for up to 48 h before carrying out uptake studies,\textsuperscript{409} to allow synthesis of new transport protein by the cells. However, as previously discussed, this procedure resulted in substantial cell loss, due to adhesion of spermatocytes to Sertoli cells. Because spermatids do not adhere to Sertoli cell monolayers to the same extent as spermatocytes,\textsuperscript{410} germ cell culture may give rise to a population enriched in spermatids.

Although cultured rat pachytene spermatocytes have been shown to sequester $[^{14}\text{C}]$MAA, cultured round spermatids do not exhibit this capacity.\textsuperscript{1} This uptake maybe an intrinsic property of the pachytene spermatocytes that is not expressed by the spermatids. However, MAA is a simple carboxylic acid that is structurally related to butyric acid, and so these findings may represent a general depression in the uptake capabilities of spermatids after isolation, with spermatocyte uptake capabilities being either less sensitive to degeneration during the isolation procedure, or being capable of regeneration after isolation. Thus, spermatid enrichment of the germ cell population by spermatocyte loss may reduce the measured uptake capabilities of the population as a whole.

A further possibility is that a component of uptake in these freshly isolated preparations is due to the presence of contaminating Sertoli cells. If the testis is capable of creatine sequestration,\textsuperscript{248} and creatine is localised within the seminiferous epithelium (Chapters 3 & 4), then presumably the Sertoli cells have such an uptake system.

Because of the above drawbacks to the experimental data presented, the use of cultured, purified spermatocytes may allow a more accurate assessment of germ cell creatine transport, and its mechanism, to be made. In addition, the uptake of creatine may allow an alternative method for the determination of germ cell survival \textit{in vitro}, since the activities of specific uptake systems have been shown to offer sensitive and selective markers for cell viability.\textsuperscript{409, 411}

\textsuperscript{1} M.W. Cook. \textit{Personal communication.}
6.4.2 The mechanism of creatine uptake into germ cells.

The energy-dependence of creatine transport into skeletal muscle and erythrocytes has been demonstrated previously. It is unlikely that this phenomenon is mediated via the trapping of creatine by phosphorylation, because PCr has been shown not to mediate the uptake of creatine into macrophages and L\textsubscript{6} myoblasts. The PCr pool in the germ cells represents only 3% of the total creatine content and therefore is unlikely to represent a significant influence upon creatine sequestration. The energy dependence of uptake however, may be a consequence of the need to maintain the sodium gradient across the plasma membrane (§1.3.2). If cells are incubated in the absence of sodium, or under conditions by which Na\textsuperscript{+} / K\textsuperscript{-}-ATPase is inhibited (reduced temperature, the presence of ouabain), the transport of sodium into the cells cannot proceed. The use of purified, cultured germ cells will enable this mechanism to be further examined, with regard to the metabolism of creatine within the testis.

6.4.3 Further studies.

The experiments described in this Chapter were merely preliminary studies using a crude germ cell preparation. Further experiments must be carried out with purified isolated germ cell types (e.g. pachytene and pre-pachytene spermatocytes, and round spermatids), in order to determine the distribution and specificity of uptake within the germ cell population.
7.1 BACKGROUND.

At the start of this project there was limited information concerning creatine within the testis, which can be summarised as follows:

a) the testis has a substantial pool of creatine,\textsuperscript{246-252}

b) the testis contains CPT activity and phosphorylates a proportion of its creatine reserve,\textsuperscript{249, 250, 252, 285, 326}

c) the testis has the capacity to synthesise creatine,\textsuperscript{251, 331}

d) damage to the testis results in an elevation in the excretion of creatine in the urine.\textsuperscript{332-335}

e) In addition, CPT activity has been implicated in spermatozoal motility for a number of species.\textsuperscript{273, 321-325}

However, the cellular distribution of creatine and localisation of the enzymes involved in its metabolism were unknown. Thus the role of creatine within the testis and its significance to testicular homeostasis remained obscure.

Moreover, since the basic understanding of testicular creatine distribution and metabolism was limited, the significance of elevated creatine excretion after damage to the organ could not be fully realised. It could not be deduced as to whether elevated creatinuria was a result of cell damage or alterations in the activities of metabolic pathways, and whether it was indicative of changes in specific cell populations or of testicular cells in general.

The aim of this project was to further explore the distribution of creatine and the enzymes involved in its metabolism within the testis as a prerequisite to the understanding of the role and significance of creatine in this organ.
7.2 THE TESTICULAR DISTRIBUTION OF CREATINE AND OF THE ENZYMES INVOLVED IN ITS METABOLISM.

Early studies in rats showed that the administration of cadmium to males induced a greater increase in the excretion of creatine than in females or orchidectomised males, and this was also the case when the animals were administered 2-ME, a toxicant that specifically damages the early and late pachytene spermatocytes. When the range of cell-specific toxicants was expanded to include Sertoli and Leydig cell cytotoxicants, an interesting pattern emerged. Creatine excretion was only elevated under conditions where damage to the cells of the seminiferous epithelium had occurred. Total destruction of the interstitial Leydig cells did not correlate with an elevation in creatine excretion. Thus, it was concluded that creatine is associated largely, if not totally, with the cells of the seminiferous epithelium (Chapter 3).

However, since the relationship between the Sertoli cells and the germ cells is extremely complex (Chapter 1), the cellular distribution within the epithelium could not be deduced from these experiments alone. Further, since the cellular distributions of enzymes involved in the pathways for creatine metabolism within the epithelium were unknown, it could not be deduced as to whether creatinuria was a result of cell leakage/lysis or altered rates of flux through different pathways for creatine metabolism.

In order to clarify these areas of confusion in vitro experiments with isolated and cultured cells were carried out.

When Sertoli cells and germ cells were isolated, it was found that both cell types had substantial stores of creatine and that when the cells were co-cultured for 24h the creatine content of the cultures increased substantially. This increase was similar to that occurring in cultures of Sertoli cells prepared from the co-cultures by removal of the germ cells with a hypotonic shock treatment. This suggested that the Sertoli cells were the major, if not sole, location of creatine synthesis within the testis.

This conclusion was confirmed by experiments in which cultured Sertoli cells and
isolated germ cells were incubated in the presence of radiolabelled precursors for creatine synthesis. The Sertoli cells incorporated the radiolabel into both creatine and its immediate metabolic precursor, GAA, whereas germ cells incubated at a similar protein density did not exhibit this activity (Chapter 4).

The secretion of creatine by Sertoli cells appears to be under the influence of intracellular cAMP levels. Both FSH and dbcAMP stimulated creatine secretion, and this effect was more pronounced in the presence of an inhibitor of cAMP catabolism (MIX). The stimulation caused by dbcAMP also appears to be dependent upon the presence of exogenous L-glutamine, and so may be the result of increased amino acid oxidation in these cells (Chapter 5).

The distribution of CPT activity within the seminiferous epithelium showed a different pattern to that of GAT and GMT. The enzyme and its creatine-derived metabolite, PCr, were found in both the Sertoli and germ cell fractions of the seminiferous epithelium (Chapter 4). This finding is supported by previous description of the presence of PCr in rat spermatocytes.†

† R. Horn, K. Purvis & V. Hansson. Unpublished results cited by Le Gac et al. 1999
7.3 A PROPOSED PATHWAY FOR INTRACELLULAR CREATINE METABOLISM WITHIN THE TESTIS.

The study of the distribution of creatine and the enzymes involved in its metabolism within the seminiferous epithelium shows that only the Sertoli cells have the capacity to synthesise creatine, but that both the Sertoli cells and germ cells contain creatine and have the capacity for its further metabolism (i.e. the reverse transphosphorylation reaction between creatine and ATP). In addition, isolated germ cells may sequester creatine by an active uptake system (Chapter 6).

These discoveries allow the postulation of an intercellular pathway for creatine metabolism with the seminiferous epithelium, whereby the Sertoli cells synthesise creatine which is in part utilised by these cells, with a fraction being released into the intercellular milieu and being sequestered by the germ cells for their own use.

The possible contribution of plasma creatine to the creatine reserve of the testis has been discussed in relation to T₃-induced creatinaemia (Chapter 4). However its significance under conditions of homeostasis in vivo is unclear. If plasma creatine was sufficient to meet testicular requirements then presumably the testis would not have developed a capacity for creatine synthesis, as is the case for skeletal and cardiac muscle and brain tissue. Therefore, although the possible role of plasma creatine has been considered in the above model it may be of limited biological significance in vivo.
7.4 THE ROLE OF CREATINE IN THE TESTIS - A HYPOTHESIS.

Theoretical models for the function of creatine have centred upon two aspects - the storage and transport of energy in the form of the high-energy phosphorus-nitrogen bond (§1.3.3).

The formation of an 'energy buffer' in the form of PCr may render cells less susceptible to anoxia since this energy reserve can be utilised when the mitochondrial phosphorylation of ADP is compromised, allowing important physiological functions to be maintained even under conditions which could not be supported by the adenine nucleotides alone.

The second proposed function of PCr is that of the creatine-PCr energy shuttle, allowing the efficient transportation of high energy phosphoryl groups from the mitochondria to specific regions within the cell. Such a system has been described in the spermatozoa of Strongylocentrotus purpuratus, and may also exist in human and chicken spermatozoa.\textsuperscript{321 - 325}

Both of these proposed functions may be of relevance in the testis since the seminiferous epithelium is maintained at a relatively low oxygen tension.\textsuperscript{397} However, it seems unusual that the interstitial Leydig cells, which are also maintained at a relatively low oxygen tension, do not appear to rely upon creatine. This difference may be related to specific functions within the cells of the seminiferous epithelium, and to the greater sensitivity of the germ cells to alterations in homeostasis.
7.5 FUTURE STUDIES.

The direction for future studies has already been discussed in detail with regard to the different aspects of the work presented (Chapters 3-6). Broadly speaking, future studies should proceed in two general directions. Firstly to establish the validity of monitoring urinary creatine excretion as a method for determining testicular damage in vivo, and secondly to further examine the metabolism and role of creatine within the seminiferous epithelium.

7.5.1 Creatinuria and testicular damage.

The experiments detailed in this thesis were carried out as part of a long-term, wide-ranging study, under the direction of Dr. J.A. Timbrell, into the validity of creatinuria as a non-invasive index of testicular damage. The results in Chapter 3 indicate that elevated creatinuria may be specifically related to damage within the seminiferous epithelium.

Further studies should be directed towards defining the spectrum of agents that induce creatinuria as a result of testicular damage. This will include examination of toxicants that act directly upon the seminiferous epithelium (e.g. cyclophosphamide) and those that damage the seminiferous epithelium indirectly (e.g. EDS). In addition, the clinical, environmental, and occupational relevance of creatinuria monitoring should be assessed by determination of human testicular creatine reserves and by measurement of creatine excretion under exposure to known toxicants (for example during cancer chemotherapy).

7.5.2 The metabolism and role of creatine within the testis.

Although the studies described in this thesis have greatly extended our knowledge of the metabolism of creatine within the testis, many questions remain unanswered. The use of [14C]creatine would allow the role of uptake from the bloodstream to be ascertained. This would enable the true importance of testicular creatine synthesis in the maintenance of testicular creatine reserves to be determined, a calculation based upon the data of Koszalka suggests that the synthesis of creatine within the testis may be of significance (§1.3.4).251
7.6 GENERAL CONCLUSIONS.

From the starting point of this project (§7.1) further conclusions can be made concerning the nature of testicular creatine in the light of the data presented in this thesis.

a) The creatine within the testis is largely limited to the seminiferous epithelium, and is distributed within both the somatic and germ cells of this structure, although its distribution within the germ cell population is unclear. Therefore it can be deduced that creatinuria arising from testicular damage is probably related to cellular damage within the seminiferous epithelium.

b) The Sertoli cells are the major, if not sole, site of creatine synthesis. The germ cells appear to have little or no synthetic activity.

c) Both the Sertoli cells and germ cells have the capacity to utilise creatine by the CPT-catalysed transphosphorylation reaction with ATP. The role of PCr in these cells remains to be clarified.

d) The germ cells appear to have the capacity to sequester creatine released from the Sertoli cells into the interstitial milieu.

In conclusion, the metabolism of creatine within the seminiferous epithelium appears to involve synthesis within the Sertoli cells, with subsequent distribution throughout the cells of the epithelium.
APPENDIX

CALCULATION OF A CONVERSION FACTOR FOR FLUORESCENCE MEASUREMENTS TO ALLOW THE DETERMINATION OF CREATINE SECRETED IN THE PRESENCE OF INTERFERING SUBSTANCES
SCEC were incubated in the presence of EMEM containing L-[guanidino-\textsuperscript{14}C]arginine for 4 h or 24 h at 32 °C. After this time the media were collected and subjected to HPLC to allow separation and quantification of the radiolabelled metabolites present.

The concentration of creatine in the culture medium after incubation for 24 h was estimated from the incorporation of radioactivity into creatine. It was assumed that all of the arginine for creatine synthesis came from the exogenous pool (i.e. the culture medium), and that the only creatine released from the cells was that which was synthesised during incubation. Under these conditions the specific activity of creatine in the culture medium would equal that of arginine, and the concentration of creatine could therefore be calculated.

Using this method the concentration of creatine in the incubation medium was estimated to be 10 μM. The only assay considered to be both practicable and sensitive enough to measure creatine at this concentration is the fluorometric method described by Conn.\textsuperscript{354} However, this assay method is not specific for creatine itself, because the reagent mixture used reacts with the guanidino group in general. Therefore potential interference from other guanidino compounds had to be taken into account.

The initial radio-metabolic studies showed that the only guanidino compounds present in the culture media from SCEC incubated for 24 h were arginine, GAA and creatine, and that these accounted for all of the radioactivity applied to the column. The only guanidino compound present in EMEM is arginine, and it was therefore considered highly likely that the only interference in the measurement of creatine would come from the presence of arginine and GAA.

These studies further showed that SCEC utilise 5.70 ± 0.66\% (mean ± SD for 3 culture dishes from a single preparation) of the arginine in EMEM over 24 h, and that the rate of utilisation over this period is linear (i.e. the extent of utilisation at 4 h was one sixth of that at 24 h, data not shown). In addition, the ratio of GAA to creatine in the medium is fairly constant, 0.72 ± 0.10 (mean ± SD for 7 culture dishes from two cell preparations).
Calculation:

Let \( F_s \) be the measured fluorescence of a sample, and let \( F_A, F_C \) and \( F_G \) be the contributions to that measured fluorescence of arginine, creatine and GAA respectively (i.e. the individual fluorescence values of the three guanidino compounds in the sample). That is

\[
F_s = F_C + F_G + F_A \quad \text{Equation A.1}
\]

\( F_A \) can be measured since this is the fluorescence of EMEM alone, and it can be determined by linear regression from the intercept of the standard curve (various concentrations of creatine in EMEM) with the ordinate, i.e. when the creatine concentration is zero (Figure 2.5).

Therefore, if the intercept is denoted \( F_o \) then

\[
F_A = F_o
\]

As arginine is utilised \( F_A \) will decrease proportionately. After 24 h, 5.7% of the arginine in the medium will have been used, and \( F_A \) will be given by the formula

\[
F_A = F_o - 0.057 F_o = F_o (1 - 0.057)
\]

Since the utilisation of arginine is linear up to 24 h, then the value of \( F_A \) at any time \( t \) (in hours) will be given by

\[
F_A = F_o \left(1 - 0.057 \cdot \frac{t}{24}\right) = F_o (1 - 0.0024 \cdot t) \quad \text{Equation A.2}
\]

The fluorescence of GAA is in the range 33-38% that of creatine, when measured on an equimolar basis. Therefore the fluorescence of GAA in the sample can be expressed in terms of that of creatine (taking 'molar fluorescence' of GAA as being 36% of that of creatine),

\[
F_G = 0.72 \times 0.36 F_C = 0.26 F_C \quad \text{Equation A.3}
\]

If Equations A.2 and A.3 are substituted into Equation A.1, then
\[ F_s = F_c + 0.26F_c + F_0(1 - 0.0024t) \]

\[ = 1.26F_c + F_0(1 - 0.0024t) \]

thus

\[ F_c = 0.79(F_s - F_0(1 - 0.0024t)) \]

Equation A.4

This is the fluorescence due solely to creatine in the sample. However, the standard curve is made up in EMEM, and so the contribution of arginine in the standard curve readings must be taken into account. Therefore, the fluorescence of the sample that will be compared to the standard curve, \( F' \), is given by the equation

\[ F' = F_c + F_o \]

Equation A.5

Combining Equations A.4 and A.5 gives

\[ F' = 0.79F_s + F_0(0.19 - 0.002t) \]

Equation A.6

In some experiments the incubation medium used was EBSS. Since EBSS does not contain arginine, then in these cases sample fluorescence was solely due to the presence of creatine and GAA, and

\[ F_o = F_a = 0 \]

Equation A.7

Substituting Equation A.7 into Equation A.6 gives

\[ F' = 0.79F_s \]

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