Mechanisms of Reactive Oxygen Species Generation by Mammalian Spermatozoa

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Contents

Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree nor is any part of it being submitted concurrently in candidature for another degree. All experiments were performed at the Medical Research Council Reproductive Biology Unit in Edinburgh, Scotland.

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Abstract

The generation of reactive oxygen species (ROS) is an activity normally associated with phagocytic leucocytes, which employ an enzymatic complex, the NADPH oxidase, to catalyze the univalent reduction of molecular oxygen to superoxide. However, it is now apparent that many other cell types, including mammalian spermatozoa, generate ROS. ROS have been implicated in peroxidative damage-associated male sub-fertility, and also, more recently in the regulation of normal sperm function. With the obvious biological importance of ROS generation by spermatozoa in mind, the aims of this PhD have been to identify and characterize the mechanisms, and cellular components involved in the generation of ROS by human spermatozoa.

Experiments investigating ROS generation by human spermatozoa have shown that the mechanisms involved are functionally similar to those pertaining to the NADPH oxidase of phagocytic leucocytes. ROS generation by human spermatozoa is enzymatic in nature and utilizes NADPH as electron donor, reducing molecular oxygen to superoxide, which subsequently dismutates to hydrogen peroxide. Further similarities with the NADPH oxidase include the probable involvement of a flavoprotein and a role for protein phosphorylation, as regulated by PKC and protein phosphatases.

Biochemical and molecular analyses of human spermatozoa have failed to detect the cellular components that form the NADPH oxidase of leucocytes in the spermatozoon. Spectral analyses of human sperm membranes failed to reveal the presence of the characteristic low potential cytochrome b_{558}, and similarly, Western blot analyses of human spermatozoa failed to detect the cytochrome b_{558}, or the presence of two of the NADPH oxidase cytosolic components, p47phox and p67phox.

ROS generating activity was extracted from human spermatozoa, using the non-ionic detergent n-octyl-D-thioglucoside. The solubilized protein extract was resolved by non-denaturing PAGE, and shown to contain 6 bands

with ROS generating activity. ROS generating activity was then isolated in an active form via 2',5'ADP affinity chromatography. The individual components of this activity were subsequently separated via SDS-PAGE, which revealed a heterogeneous protein population. One of the proteins was purified, and a polyclonal antibody raised against it. The protein had a molecular weight of 55kDa, and the antibody raised against it cross reacted with a band on non-denaturing PAGE gels capable of ROS generation. Western blot analyses carried out employing the anti-55kDa polyclonal antibody, revealed donor-donor variation in the expression of this protein in spermatozoa, and also that it was widely expressed, in various forms, in somatic tissues. Of all the non-human spermatozoa investigated, the antibody only cross-reacted with the spermatozoa of the rat. Indirect immuno-fluorescent staining of spermatozoa revealed the 55kDa protein to be located over the entire surface of the spermatozoon, with the mid-piece, acrosome and distal tail region being particularly heavily stained. The antibody did not have any effect on ROS generation by human spermatozoa.

In order to identify the 55kDa protein, a human testicular cDNA library, cloned into the expression vector λgt 11, was screened with the antibody. One positive clone was identified. The DNA insert from this clone was amplified using the polymerase chain reaction, and was shown to be approximately 1.9kB in length. The insert was sub-cloned into a pCR plasmid vector and then partially sequenced. Preliminary sequence data obtained from this clone has indicated that the 5' end of the insert shows significant nucleotide sequence homology with the α-2 subunit of human collagen VI.

Satellite studies were conducted investigating cross-species generation of ROS by spermatozoa This work revealed that the ability to generate ROS, employing NADPH as electron donor, is widespread; rat, hamster, guinea pig, and mouse sperm all generating ROS in response to exogenous NADPH.

In summary, the work presented in this thesis has shown that mammalian spermatozoa possess a specialized mechanism for ROS generation. This mechanism, though sharing some functional properties of the NADPH oxidase of phagocytic leucocytes, is structurally distinct. It remains for the

'oxidase' of human spermatozoa to be conclusively identified, but hopefully the information supplied in this thesis will enable this in the very near future.

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Abbreviations

2', 5' ADP	2′,5′ adenosine diphosphate
AEBSF	4-(2-aminoethyl)-benzenesulphonylfluoride
AMPS	ammonium persulphate
ANOVA	analysis of variance
AR	acrosome reaction
b.p.	base pair
BCA	bicinchoninic acid
BSA	bovine serum albumin
BWW	Biggers, Whitten and Whittingham medium
cAMP	cyclic adenosine monophosphate
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cDNA	complementary deoxyribonucleic acid
CGD	chronic granulomatous disease
cGMP	cyclic guanosine monophosphate
CHAPS	3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate
CHAPSO	3-(3-cholamidopropyl)dimethyl-ammonio-2-hydroxy-1-propanesulfonate
CPK	creatine phosphokinase
CR	cortical reaction
CRS	control rabbit serum
dATP	deoxyadenosine triphosphate
DCIP	2,6, dichloroindophenol
dCTP	deoxycytidsine triphosphate
dGTP	deoxyguanosine triphosphate

DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPI	diphenylene iodonium
dTTP	deoxythymidine triphosphate
ECL	enhanced chemiluminescence
EDTA	disodium ethylenediaminetetra acetate
ELISA	enzyme-linked immunosorbant assay
FAD	flavin adenine dinucleotide
FCS	foetal cord serum
FITC	fluorescein isothiocyanate
FLSD	Fishers least significant difference
FMLP	N-formyl-methionyleucylphenylalanine
FPLC	fast performance liquid chromatography
FSH	follicle stimulating hormone
G6PDH	glucose-6-phosphate dehydrogenase
GTP	guanosine triphosphate
H7	1-[5-isoquinolinesulfonyl]-2-methylpiperazine
HDL	high density lipoprotein
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane-sulfonic acid)
HEPT	hamster egg penetration test
HIV	human immunodefficiency virus
HMS	hexose monophosphate shunt
HRP	horseradish peroxidase
IEF	isoelectric focusing
IgG	immunoglobulin G
IPTG	isopropyl-1-thio-ß-D-galactoside
kb	kilo base

kDa	kilo Daltons
LB	Luria-Bertani
LDH	lactate dehydrogenase
LH	luteinizing hormone
MRB	membrane resuspension bufffer
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
NAD	nicotinamide adenine dinucleotide (non-reduced)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate (non-reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NBD	nitroblue diformazan
NBT	nitroblue tetrazolium
NGS	normal goat serum
NOS	nitric oxide synthase
OA	Okadiac acid
OG	n-octyl-ß-D-glucoside
OTG	n-octyl-ß-D-thioglucoside
PAGE	polyacrylamide electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PKM	protein kinase M

PMS	phenazine methosulfate
PMA	phorbol 12-myristate 13-acetate
PMB	plasma membrane block
PMSF	phenylmethyl sulphonyl fluoride
PP	protein phosphatase
PVA	polyvinyl alcohol
PVDF	polyvinylene difluoride
RNA	ribonucleic acid
ROS	reactive oxygen species
S.E.	standard error
SDS	sodium dodecyl sulphate
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris-borate-EDTA buffer
TBS	Tris-buffered saline
TBST	Tris-buffered saline-Tween 20
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)-methylamine
Tween 20	polyoxyethylenesorbitan monolaurate
UV	ultra-violet
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Chapter 1

Introduction

In view of the rising prevalence of male infertility and the growing interest in engineering new, more effective, contraceptive strategies, a knowledge of the cellular, biochemical, and molecular events that regulate the fertilizing potential of human spermatozoa is highly desirable. One area of research where some progress has been made in recent years, is in defining the importance of reactive oxygen species (ROS) in the stimulation and suppression of mammalian sperm function.

For many years it has been known that normal oxygen metabolism produces ROS (Halliwell and Gutteridge, 1986), and it is also known that some cells possess specialized systems for the specific, and co-ordinated generation of these molecules. Up until only a few years ago, it was a widely held belief that only phagocytic leucocytes possessed such a system, and that ROS were only biologically relevant in the pathological conditions associated with infection. However, in recent years, it has become increasingly acknowledged that the possession of ROS generating systems is not restricted to professional phagocytes, and that such systems, and the molecules they generate, are probably very important modulators of normal cell function. It is now apparent that many cell types possess specific, enzymatic mechanisms for the generation of ROS (Cross and Jones, 1991), e.g. endothelial cells, mesangial cells, fibroblasts, thyroid cells, Epstein-Barr transformed B lymphocytes, and adipocytes to name only a few, although it is seldom realized that the first cell type in which ROS generation was described was the human spermatozoon (MacLeod, 1943). Over fifty years ago the andrologist John MacLeod presented the first data which suggested that human spermatozoa generated ROS (MacLeod, 1943), and this work has been latterly supported with direct

evidence, showing that human spermatozoa and those of other mammalian species do indeed generate and release ROS (Tosic and Walton, 1946; Holland *et al*, 1982; Alvarez and Storey, 1984; Alvarez et al, 1987; Aitken and Clarkson, 1987a and b; Bize and Sharpe, 1990; Kumar *et al*, 1990).

ROS as their name suggests, are highly reactive molecules and thus can profoundly alter cell status through their actions on the lipid-rich, plasma membrane of the cell, and on other cellular components, e.g. enzymes and DNA (Halliwell and Gutteridge, 1986; Cochrane *et al*, 1987). With this in mind, it is hardly surprising that it has been widely reported in recent years, that the genesis of ROS by mammalian spermatozoa has profound effects on their function, both normal and abnormal (for recent reviews see Aitken and Fisher, 1994; Aitken 1994a and b; de Lamirande and Gagnon, 1995). The realization that ROS generation is involved in the physiological and pathophysiological events associated with sperm function, has made this phenomenon a highly attractive target for research. This is, in part, due to concern over the rising prevalence of male factor infertility (Cummins *et al*, 1994), and to the desire to devise more effective contraceptive strategies (Edwards, 1994). To address either of these aspects of reproductive biology, an in-depth knowledge of the biochemical mechanisms underlying sperm physiology is required. Thus, the main aims of this thesis were to explore the biochemical mechanisms underlying the ability of mammalian spermatozoa to generate ROS, and to identify sperm molecules involved in this phenomenon.

Currently, although it has been categorically shown that human spermatozoa generate ROS (Alvarez et al, 1987; Aitken and Clarkson, 1987a and b), the mechanisms by which they do so have not been fully elucidated. It does appear that the cellular system employed by human spermatozoa for ROS generation may be similar to that employed by phagocytic leucocytes, i.e. the NADPH oxidase, although this has not been experimentally proven. In this

thesis, information that is already available on the regulation of leucocyte NADPH oxidase activity has been exploited, to ascertain whether the ROS generating system in human spermatozoa bears any similarities with the leucocyte NADPH oxidase. The purpose of such studies is ultimately to characterize the spermatozoon's capacity for free radical generation at a molecular level and to determine the factors that regulate it's activity. Once in possession of such knowledge, therapeutic strategies could possibly be devised to diminish ROS-associated male sub-fertility, and also disruptive techniques developed, that would target this phenomenon for contraceptive purposes.

In the following chapters of this thesis, I shall review the current state of our knowledge relating to fertilization as a whole, along with that pertaining to ROS generation and sperm function. Subsequent to this, experimental studies designed to elucidate the biochemical basis of ROS generation by human spermatozoa will be described, along with those aimed at identifying sperm molecules involved in this phenomenon. The final experimental chapter of this thesis will explore ROS generation by various mammalian species, and attempt to define the ontogeny of this phenomenon during the testicular differentiation of precursor germ cells and the subsequent maturation of spermatozoa in the epididymis. Finally, the last chapter of this thesis will take the form of a general discussion, bringing together all the work presented in this thesis in order to draw the appropriate conclusions and examine the future direction that research in this area might take.

Chapter 2

General background and review of the literature

2.1 Introduction

This thesis unites two fairly disparate areas of biological research, i.e. mammalian fertilization and the cellular genesis of reactive oxygen species. Considerable research by many workers in the biological and biochemical sciences has lead to the accumulation of a vast amount of information pertaining to each of these areas. It therefore seems appropriate to initiate this thesis with a review of this information, in order to create an informed, balanced platform on which to base the proceeding discussion.

2.2 Fertilization

Sexual reproduction is characterized by the union of haploid male and female gametes during the process of fertilization. This thesis will not deal with the gross, morphological and behavioural differences between males and females, but it is important to appreciate the crude differences between the gametes of the two sexes. In all animals, and even plants, the basic difference between the sexes is the size of their gametes; females produce large, immobile, food rich gametes - eggs, while male gametes, spermatozoa, are tiny, highly motile cells which have been described as consisting of little more than a piece of self-propelled DNA. Reproduction involving the fusion of gametes of unequal size is called *anisogamous* sex, and is thought to have evolved from *isogamy* (involving gametes of equal size) by an evolutionary process in which smaller than average gametes successfully parasitised those that were larger than average. This eventually lead to the two specializations we see today, i.e. small, active spermatozoa, and large, immotile eggs. (Parker *et al*, 1972)

Due to the haploid nature of gametes and the recombination of genetic material which takes place during meiotic stages of gametogenesis, when fusion occurs between the two different, parental gametes a new, unique individual is formed, and with the creation of such individuals, the evolution and destiny of a species may be re-cast. Due to the fundamental importance of fertilization, its biology has been studied, at various levels of resolution, since antiquity. Indeed, as long ago as the seventeenth century, the egg and embryo were observed by de Graaf, though the first follicular egg was not observed until 1827, by von Baer (Austin and Bishop, 1957). Malpighi, in 1673, suggested that the egg of the hen contained an immature chick, which was subsequently stimulated, by the spermatozoon, to mature and develop (Gwatkin, 1977). In 1677, spermatozoa were first seriously studied, by Leeuwenhoek, and he hypothesised that the spermatozoon, alone, developed into the embryo, after entry into the egg. However, it was not until almost 200 years later, in 1876, that Hertwig realised that the nucleus of the fertilized egg was formed by the nuclei of both the egg and spermatozoon, via their union. This fundamental development in reproductive biology spurred the biologists of the time into rapid and highly productive research and only a year later, in 1877, Fol reported that the egg and spermatozoon underwent some process in which the normal somatic chromosome number was reduced to half. Fol realized that the male and female pro nuclei (as they were named) both contributed to the groups of chromosomes present in the fertilized egg, and that it was the intermingling of these groups of chromosomes that resulted in equal maternal and paternal contribution of hereditary factors, and the restoration of the diploid chromosome number. Thus, the modern concept of fertilization was established (Austin and Bishop, 1957).

Of the more contemporary research into fertilization, that before 1950 tended to be carried out using invertebrate models, e.g. the sea urchin, and was thus not always strictly applicable to mammalian fertilization. However,

since 1950, research into mammalian fertilization has progressed quite rapidly due, in the main, to the availability of mammalian gametes, and the development of mammalian *in vitro* fertilization technology, first demonstrated in the rabbit by Douzier and colleagues in 1954 (cited in Austin and Bishop, 1957), and by Chang in 1959 (Chang, 1959). The recent advances in our knowledge of fertilization have been very impressive, and in some areas of fertilization research, the research carried out with mammalian gametes is way ahead of its invertebrate counterpart (Yanagimachi, 1994).

The union of the spermatozoon and egg, which signifies the end point of fertilization, involves a series of signalling mechanisms which lead to species-specific gamete interactions, resulting in the activation and fertilization of an egg by a single spermatozoon. The biochemical pathways and molecular events comprising the fertilization process, involving gamete recognition, activation and fusion are very complex. However, with the numerous scientific tools currently available to the gamete biologist, the discrete pathways, and regulatory events that shape fertilization are now beginning to be clarified. The current opinions and theories abounding in this scientific arena will now be discussed, with particular reference to the role of the spermatozoon in fertilization.

2.2.1 The spermatozoon

The spermatozoon, although being the smallest cell in most organisms, is a highly differentiated, specialized cell, which undergoes profound morphological, physiological and biochemical changes during its' development and maturation. Spermatozoa are produced in the seminiferous tubules of the testis, by a process known as **spermatogenesis** (de Kretser and Kerr, 1988; Steinberger, 1989). Spermatogenesis, in humans, does not begin until the male reaches puberty, the pituitary gland begins to secrete luteinizing hormone (LH), which stimulates the Leydig cells, in the testis, to secrete

testosterone and follicle stimulating hormone (FSH) (Steinberger, 1971; O'Dell, 1989; Hall, 1988; Steinberger and Steinberger, 1989). It is testosterone and FSH that stimulate spermatogenesis, through their combined action on Sertoli cells (Tindall *et al,* 1985; Bardini *et al,* 1988; Steinberger, 1989; Steinberger and Steinberger, 1989; de Kretser and Kerr, 1988). Sertoli cells completely envelop the developing male gamete, protecting and nourishing it, whilst orchestrating its development by a complex process that is not yet not fully understood.

Spermatogenesis

Spermatogenesis itself, involves cell division, cell proliferation and subsequent cell differentiation. Early in embryogenesis, primordial germ cells migrate into the testis and develop into immature germ cells, **spermatogonia** (de Kretser and Kerr, 1988; Steinberger, 1989). At sexual maturity the spermatogonia, located on the outer edge of the seminiferous tubules, proliferate via mitosis. Some of the daughter spermatogonia stop proliferating and differentiate into **primary spermatocytes**. These cells enter the first meiotic prophase, during which time genetic recombination takes place. The cells then proceed with division I of meiosis to produce two **secondary spermatocytes**. Each secondary spermatocyte contains 22 autosomal chromosomes and an X or Y chromosome. Each chromosome still consists of 2 sister chromatids at this point, but this situation changes when the cells undergo meiotic division II to produce 4 **spermatids**, each with a haploid number of single chromosomes.

Morphological differentiation of spermatids then takes place, a process known as **spermiogenesis**, which involves cytoplasmic remodelling and extrusion, with the concomitant development of the tail, mid-piece, and the acrosome (de Kretser and Kerr, 1988). Briefly, the immature, round spermatid moves towards the lumen of the tubule and, as it does so, it elongates and the Sertoli cell cytoplasm, surrounding the spermatid, retracts. The elongate spermatids undergo further modification and are eventually released into the

lumen of the seminiferous tubule as immature spermatozoa, from where they are released into the efferent ducts of the testis. From the efferent ducts, the spermatozoa pass through the epididymis where, in mammalian species, they undergo further maturational events.

Epididymal maturation of spermatozoa

It was over 80 years ago, in 1913, that it was first noticed that spermatozoa from different regions of the epididymis showed different characteristics, and it was at this time that Tournade proposed that spermatozoa under went a 'ripening' process during their epididymal passage (cited in Bedford, 1967). However, it was not until over 50 years later that the principle of epididymal maturation was refined, and experimental evidence obtained to support such a hypothesis (Bedford, 1967). It is now clear that important changes take place in the epididymis of most mammals, including man, that confer upon spermatozoon the potential to fertilize eggs. The testicular spermatozoa of most mammalian species have not undergone such changes, and do not possess the ability to move progressively, or to interact with the egg (Bedford, 1975). Spermatozoa gain these abilities, to some extent, as they pass through the epididymis. The spermatozoa, at this point, have lost most, if not all, ability for protein and lipid biosynthesis, but they are equipped to modify and degrade existing molecules (Bedford and Hoskins, 1990). The biochemical environment generated in the epididymis clearly has a profound effect on the functional status of the maturing spermatozoon (Moore *et al*, 1989; Cooper, 1990). During their passage through the epididymis, spermatozoa undergo many morphological and biochemical changes that enable them to successfully traverse the female reproductive tract, recognise, bind and then fuse with the egg (Amann *et al*, 1993). The changes that the spermatozoon undergoes in the epididymis are not merely the result of a passive ageing process, but are an actively orchestrated series of carefully regulated cellular events (Robaire and

Hermo, 1988). The changes the spermatozoon undergoes include alterations in the metabolism of the spermatozoon (Mann and Lutwak-Mann, 1981); changes in its movement characteristics (Acott *et al,* 1983; Yeung *et al,* 1994); changes in its zona binding ability (Saling, 1982; Moore *et al.,* 1983; Moore, 1990); changes in intracellular ions (Vijayyaraghavan and Hoskins, 1989); and numerous plasma membrane associated changes. The plasma membrane associated changes include changes in its' phospholipid distribution (Hall *et al,* 1991; Rana *et al,* 1993); changes in its' surface charge, becoming increasingly negatively charged as the spermatozoon passes through the epididymis (Bedford, 1963; Bedford *et al,* 1973; Moore, 1979); and changes in protein glycosylation (Tulsiani *et al,* 1993). Another sperm maturation event occurring in the epididymis is the formation of structurally stabilizing disulphide bonds, in the nucleus and tail of the spermatozoon (Calvin and Bedford, 1971).

Although the maturational events that occur during epididymal transit equip the spermatozoa for active movement, and are essential for the spermatozoa to realize their fertilizing potential, they are still not immediately capable of fertilization (Drobnis, 1993). The final functional maturation of the spermatozoon occurs after the spermatozoa have left the male reproductive tract, and have entered the reproductive tract of the female, as discussed below.

2.2.2 Spermatozoa in the female reproductive tract

Between 50 and 300 million sperm are ejaculated during mammalian coitus, the actual number being dependent on the species (Wassarman, 1987a). Of these, only a tiny minority successfully traverse the female reproductive tract and actually reach the site of fertilization (Cummins and Yanagimachi, 1982; Drobnis and Overstreet, 1992). However, the structural and functional attributes that the mammalian spermatozoon requires to achieve fertilization are not, generally, all present at the time of ejaculation. The cells must first

undergo further maturational events, within the reproductive tract of the female, before they acquire the ability to fertilize an oocyte (for reviews see Wassarman, 1987a and b; Zaneveld *et al*, 1991; Drobnis, 1993; Yanagimachi, 1994). The changes that the spermatozoon undergoes in the female reproductive tract are collectively termed **capacitation**, and usually take place during sperm transport to the site of fertilization, i.e. usually within the uterus and fallopian tubes of the female (Drobnis, 1993). Once in the vicinity of the oocyte, the spermatozoon must undergo further changes, the trigger for which is an exocytotic event, called the **acrosome reaction** (AR). A crude, diagrammatic representation, of the events involved in fertilization is depicted in figure 2.1.

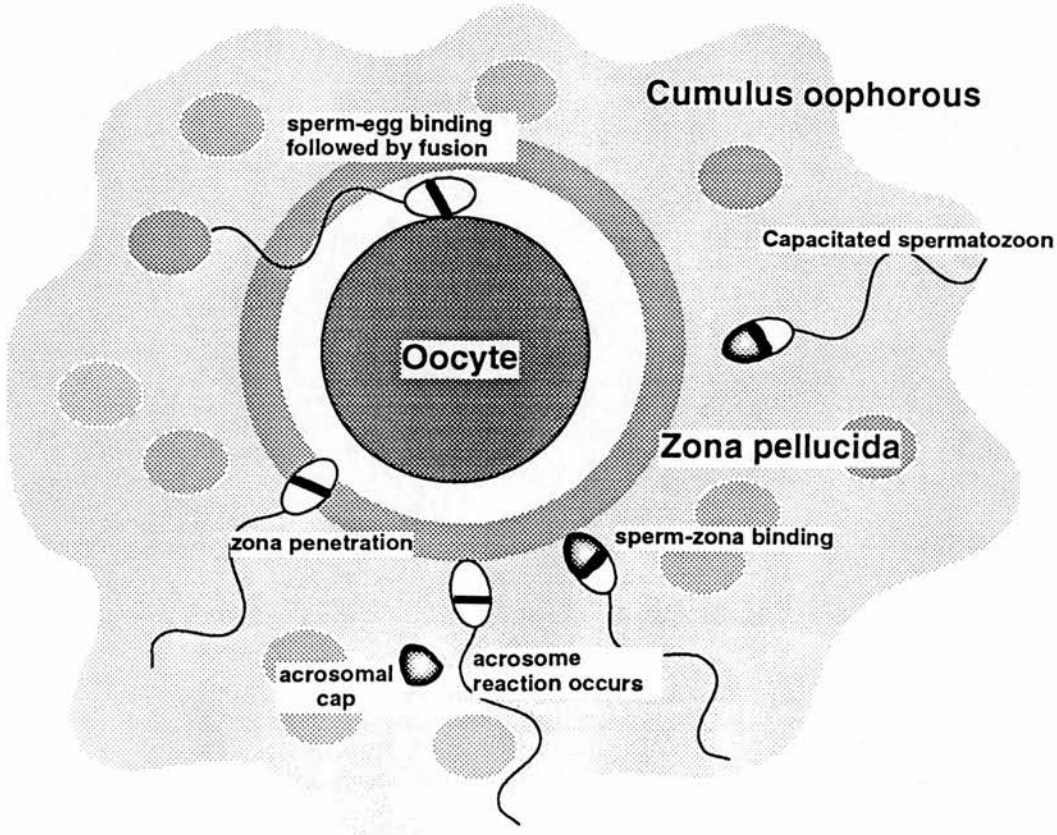

Figure 2.1 Diagrammatic overview of the events that take place during mammalian fertilization. For a more in-depth description see text.

The changes which occur as a consequence of the AR, then allow the spermatozoon to penetrate the zona pellucida and initiate fusion with the

by modifying its outer investments to prevent any additional spermatozoa from fusing with, what is now, a zygote. The events mentioned above will now be described in greater detail.

2.2.3 Capacitation and the acrosome reaction

The need for further sperm maturation, or capacitation, after ejaculation but prior to fertilization, was first realised in the late 1940's as a result of unsuccessful attempts to fertilize eggs with testicular and epididymal spermatozoa (Noyes, 1953). Noyes and colleagues, discovered that only spermatozoa which had resided in the oviducts of donors for a few hours, were capable of *in vitro* fertilization, although the importance and significance of the findings was not fully appreciated at this point, by Noyes and colleagues.

The requirement for capacitation was first, fully recognized in the 1950's, independently by Chang (1951) and Austin (1951), in the rabbit and rat respectively, but even now, over forty years later, the molecular basis for capacitation is still not fully understood, though some headway is beginning to be made as outlined below.

Capacitation

The term 'capacitation' was first coined by Austin (Austin, 1952), to describe the process by which spermatozoa gain the ability to fertilize oocytes, and it is often viewed as the cellular changes that enable the spermatozoon to undergo the acrosome reaction (Drobnis, 1993; Yanagimachi, 1994). The changes that occur during capacitation have been one of the major focal points of researchers studying fertilization, and knowledge is now rapidly accruing as to the cellular processes involved.

During capacitation the molecular composition and physical properties of the sperm surface change (Drobnis, 1993; Yanagimachi, 1994). When spermatozoa are first ejaculated and are suspended in seminal plasma, they

become coated with seminal plasma proteins (Dacheux *et al*, 1989; Jones, 1989). Among these sperm-coating proteins are so called 'decapacitation' factors (Eng and Oliphant, 1978). Two such mammalian decapacitation factors, shown to reversibly inhibit fertilization, are acrostatin, a 5kDa glycoprotein, that inhibits acrosin; and a high molecular weight polyatosaminyl glycoside, that inhibits galactosyltransferase, an enzyme thought to be involved in zona binding (Fraser, 1990; Zaneveld *et al*, 1991). An important part of the capacitation process is the removal of such decapacitation factors (Eng and Oliphant, 1978; O'Rand, 1982).

Other plasma-membrane-associated changes also take place during capacitation including the redistribution and loss of sperm surface components (Yanagimachi, 1981; Yanagimachi, 1994). In many mammalian species, including humans, the pattern of lectin binding on the sperm surface changes during capacitation, indicating that surface carbohydrates have been lost, modified, unmasked, and/or rearranged (Cross and Overstreet, 1987; Talbot and Chacon, 1981; Koehler, 1981). One carbohydrate sperm component that is thought to be lost during capacitation is sialic acid, and its removal is thought to be associated with the development of zona-recognition (Zaneveld *et al*, 1991; Lassalle and Testart, 1994). Loss and/or modification of such carbohydrates from the surface of the spermatozoon also results in a decrease in the net negative charge of the cell during capacitation (Langlais and Roberts, 1985; Zaneveld *et al*, 1991).

Other sperm molecules that undergo changes, are the integral membrane proteins of the lipid bilayer (Tesarik, 1984). In this context, an extensively studied protein which undergoes topographical rearrangement during capacitation is PH20, a guinea pig antigen that is postulated to be the sperm zona receptor of this species (Myles *et al*, 1990). This antigen, during capacitation and the acrosome reaction, migrates from the plasma membrane overlying the posterior head of the spermatozoon, to the inner acrosomal

membrane (Primakoff *et al*, 1987). Human sperm surface molecules which also show capacitation-dependent changes in their distribution, include fibronectin-like molecules (Fusi and Bronson, 1992).

Another membrane-associated change which the spermatozoon undergoes during the capacitation process is an increase in membrane fluidity and permeability (Langlais and Roberts, 1985; Zaneveld *et al*, 1991; Kurpisz, 1993). The lipid composition of the sperm plasma membrane changes significantly during capacitation (Langlais and Roberts, 1985; Wolf *et al*, 1986; Benoff, 1993). Such changes probably lead to the establishment of potential fusogenic domains in the plasma membrane, and include the modification of membrane lipids (Drobnis, 1993), e.g. methylation of phospholipids (Llanos and Meizel, 1983; Meizel, 1985), and the loss of cholesterol from the membrane (Langlais and Roberts, 1985; Benoff, 1993). A decrease in the ratio of cholesterol to phospholipid (C/P) in the plasma membrane, renders it more permeable and fusogenic, and is thought to be directly involved in the capacitation process (Langlais and Roberts, 1985; Benoff, 1993; Drobnis, 1993; Yanagimachi, 1994). Cholesterol loss from the spermatozoon is enabled by cholesterol acceptor molecules present in the reproductive tract of the female, e.g. HDL (high-density lipoprotein) and apolipoprotein A (Ravnik *et al*, 1990; Parks and Ehrenwald, 1990; Langlais and Roberts, 1985). These acceptor molecules take up cholesterol from the surface of the spermatozoon. It has been shown that human follicular fluid contains a component, lipid transfer protein (LTP-1), that stimulates capacitation (Ravnik *et al*, 1992), and it is likely that this factor is acting via a mechanism similar to that described above, i.e. through the removal of cholesterol. Albumin is also a cholesterol acceptor molecule, and it is thought that its ability to support *in vitro* capacitation is linked to this property (Go and Wolf, 1985; Ravnik *et al*, 1993).

Along with the plasma membrane associated changes, other fundamental changes also occur during capacitation, which collectively result

in alterations to the movement characteristics of the spermatozoon. The acquisition of the capacitated state, in many species, is indicated by a shift from the linear swimming trajectories exhibited by spermatozoa upon ejaculation, to a non-progressive form of movement characterized by high amplitude, asymmetrical flagellar waves. This form of movement is termed '**hyperactivation**' (Yanagimachi, 1981) and was first described by Yanagimachi, in the hamster (Yanagimachi, 1969; Yanagimachi, 1970). Hyperactivated movement is thought to be essential for zona penetration (Katz and Yanagimachi, 1981; Kurpisz, 1993; Drobnis, 1993; Yanagimachi, 1994), and has been shown to occur in mice (Fraser, 1977); rats (Shalgi and Phillips, 1988); dogs (Mahi and Yanagimachi, 1976); rabbits (Cooper *et al*, 1979) and humans (Morales *et al*, 1988).

Other capacitation-related modifications which occur are metabolic changes, e.g. increased glycolytic activity and oxygen consumption (Boell, 1985; Fraser and Ahuja, 1988); changes in intracellular ion concentrations, e.g. an increase in the concentration of intracellular calcium (Lindemann and Goltz, 1988; Tash, 1989; Fraser and McDermott, 1992; DasGupta *et al*, 1993); and an increase in cAMP levels (Stein and Fraser, 1984; Berger and Clegg, 1983; White and Aitken, 1989; Duncan and Fraser, 1993). To some extent, a rise in cytoplasmic calcium levels during capacitation may be responsible for the changes in flagellar movement via calmodulin-dependent mechanisms including the increase in sperm cAMP levels (Berger and Clegg, 1983; Stein and Fraser, 1984; White and Aitken, 1989). Such changes ultimately result in alterations in protein phosphorylation, which has been shown to be an important part of the capacitation process, including hyperactivation (Tash, 1989; Duncan and Fraser, 1993; Furuya *et al*, 1993a and b).

Thus, the capacitation process, equips the cell to participate in the subsequent steps necessary for fertilization. Specifically, once capacitated, the spermatozoon is primed to undergo the next crucial cellular event involved in

the fertilization process, i.e. the acrosome reaction. This event is described below.

The acrosome reaction

The **acrosome** is a membrane bound, cap-like organelle, biochemically analogous to a secretory granule, that appears during spermatogenesis as a product of the Golgi apparatus. In all mammalian species, it occupies the anterior region of the sperm head just above the nucleus and beneath the plasma membrane (Figure 2.2) (Wassarman 1987a and b). The acrosome contains a variety of hydrolytic and proteolytic enzymes, including acrosin and hyaluronidase. These enzymes are present in the acrosome itself and are also bound to the inner acrosomal membrane (Zaneveld *et al*, 1991).

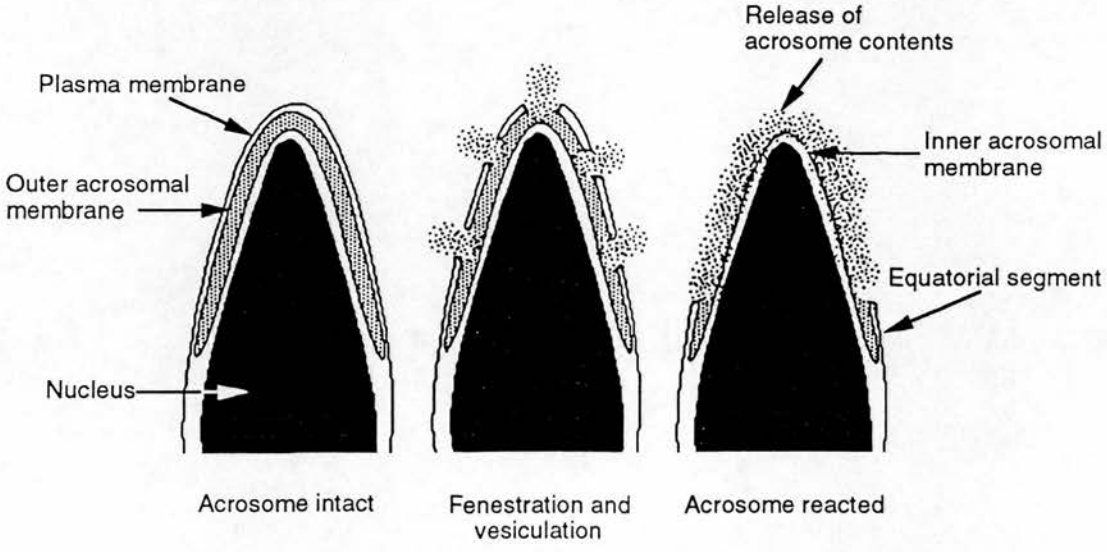

Figure 2.2 Schematic representation of the acrosome reaction.

The **acrosome reaction** (AR) is an exocytotic event, involving multiple fusion's between the plasma membrane and outer acrosomal membrane of the spermatozoon at many sites (Wassarman 1987a and b; Drobnis, 1993). This results in the fenestration and vesiculation of the membranes, as a consequence of which, the acrosomal contents are released. The acrosome reaction is a prerequisite for successful fertilization, since it is necessary for the

spermatozoon to be able to successfully traverse the outer investments of the oocyte, and to recognise and fuse with the vitelline membrane of the egg (Yanagimachi, 1994).

Due to the presence of hyaluronidase in the acrosome, and the presence of its substrate, hyaluronic acid, in the acellular matrix of the cumulus oophorous, it was thought that the main role of the AR was to facilitate the passage of the spermatozoon through this structure (McRorie, and Williams, 1974). However, it is now known that non-acrosome reacted spermatozoa can successfully navigate the cumulus and bind to the zona pellucida (Saling *et al*, 1979; Talbot, 1985; Cherr *et al*, 1986). In fact is has been thought for some time that hyaluronidase activity is bound to the surface of the spermatozoon (Lewin *et al*, 1982; Talbot, 1985; Zao *et al*, 1985), and it is now apparent that the sperm plasma membrane, of humans and guinea pigs at least, possesses a surface component, PH-20, with hyaluronidase-like activity. This activity is sufficient to enable penetration of the cumulus cell layer surrounding the oocyte, and thus, negates the need for acrosomal hyaluronidase to digest the extracellular matrix of the cumulus (Gmachl *et al*, 1993; Lin *et al*, 1994). It is probable that the cumulus, whilst not the primary stimulus for the induction of the AR, does play a fundamental role in supporting sperm capacitation, and causes the modification of the acrosome in some way (Gwatkin *et al*, 1972; Gwatkin *et al*, 1974; Bavister, 1982; Fukui, 1990). It has also been postulated that the cumulus acts as a selection device, filtering out morphologically and functionally, abnormal spermatozoa (Carrell *et al*, 1993).

It is also postulated that the AR may occur not only on contact with the cumulus and the surface of the zona, but elsewhere in the female reproductive tract, where spermatozoa come into contact with other AR inducing factors. Three of the main, known possible physiological, inducers of the acrosome are the cumulus oophorous (Siiteri *et al*, 1988; Stock *et al*, 1989; Carrell *et al*, 1993), the zona pellucida (Saling *et al*, 1979; Bleil and Wassarman, 1983; Wassarman *et*

al, 1986; Lee *et al*, 1992; Bielfeld *et al*, 1994), and follicular fluid (Tesarik, 1985; Suarez *et al*, 1986; Morales *et al*, 1992). It is possible, that due to the fundamental importance of the AR in fertilization, and hence in the propagation of species, all three inducers are important *in vivo*, acting as biochemical safety mechanisms, or in synergy, ensuring that the AR does occur, in the vicinity of the oocyte and thus promote fertilization (Zaneveld *et al*, 1993). However, evidence is accumulating to suggest that the most important inducer of the AR, *in vivo*, is indeed the zona pellucida, and that the occurrence of the AR at this specific location, is vital for successful fertilization (Drobnis and Overstreet, 1992; Drobnis, 1993, Yanagimachi, 1994).

It is now thought that the physiological AR occurs in response to a direct trigger, i.e. a biochemical signal such as the binding of a specific ligand to a sperm receptor (Saling *et al*, 1979). Two such ligand inducers which have been well characterized are ZP3, one of the 3 glycoproteins comprising the zona pellucida (Bleil and Wassarman, 1983; Florman *et al*, 1984; Wassarman *et al*, 1986; Moller *et al*, 1990; Kinloch *et al*, 1991; Beebee *et al*, 1992; Van Duin *et al*, 1994), and the steroid hormone, progesterone, present in follicular fluid and secreted by the cells of the cumulus oophorous (Morales *et al*, 1992; Tesarik *et al*, 1992; Uhler *et al*, 1992; Blackmore, 1993; Saaranen *et al*, 1993; Wistrom and Meizel, 1993; Blackmore *et al*, 1994). Numerous signalling pathways may be involved in inducing the AR, dependent upon the ligand involved, but they all ultimately result in an increase in protein phosphorylation (De Jong *et al*, 1991; Rotem *et al*, 1992) and/or a rise in intracellular calcium concentration (Yanagimachi, 1981; Fraser, 1987; Florman *et al*, 1989; Roldan and Flemming, 1989, Fraser, 1993; Florman, 1994). Some of the possible signal transduction pathways that have been described, include the adenylate cyclase, cAMP, protein kinase A pathway; the phospholipase C, diacylglycerol. protein kinase C pathway, and the guanylate cyclase, cGMP, protein kinase G pathway (Zaneveld *et al*, 1993). It is also thought that G proteins may be involved in the

induction of the AR (Lee *et al*, 1992; Ward *et al*, 1994), and that tyrosine phosphorylation also plays an important role in this phenomenon (Naz and Ahmad, 1991; Leyton *et al*, 1992). Leaving aside the signal transduction pathways involved in the induction of the AR, the general cellular prerequisites for the AR appear to be concomitant influxes of sodium and calcium, an efflux of hydrogen ions, and a simultaneous increase in intracellular pH (Yanagimachi, 1994). These changes in the intracellular milieu of the spermatozoon ultimately result in the induction of the AR, and the generation of a fusogenic plasma membrane, capable of recognizing, and fusing with, the vitelline membrane of the oocyte.

2.2.4 Sperm-oocyte interaction

The development of expression systems for human recombinant ZP3 (Kinloch *et al*, 1991, Beebee *et al*, 1992; Van Duin *et al*, 1994), have enabled significant advances in the study of the human sperm acrosome reaction, and in the cellular mechanisms involved in the interaction of the spermatozoon with the ZP3 ligand. Hopefully, very soon, a definitive model of the receptor activating signal transduction mechanisms set in motion following sperm contact with the zona pellucida, will emerge. In fact, a model is already beginning to emerge, involving receptor cross-linking and aggregation on the sperm surface, induced by the zona glycoprotein, ZP3 (Leyton and Saling, 1989). The aggregation subsequently induces autophosphorylation of the receptor, a result of the activation of its' intrinsic tyrosine kinase activity (Leyton and Saling, 1989a and b; Leyton *et al*, 1992). The ZP3-induced phosphorylation of the receptor then leads to the activation of a cascade of second messenger systems involving changes in intracellular calcium and pH (as described above) and, ultimately, the induction of the AR. Most of these studies have been conducted using the mouse as an animal model, but preliminary data, with respect to the progesterone-induced AR, is beginning to point to a similar

situation existing in the human (Tesarik *et al*, 1992; Tesarik and Mendoza, 1992; Tesarik *et al*, 1993; Foresta *et al*, 1993; Blackmore, 1993; Turner *et al*, 1994; Blackmore *et al*,, 1994).

Once the AR has occurred, it is probable that hyaluronidase, released locally, digests the cumulus matrix around the sperm flagellum, allowing it to move more freely and vigorously, thus aiding zona penetration (Drobnis *et al*, 1988). Other enzymes released from the acrosome may also aid zona penetration, e.g. acrosin (Drobnis, 1993), and this enzyme may also participate in post-translationally modifying sperm adhesion molecules, e.g. the proteolytic modification of PH30, thus facilitating secondary zona binding and/or sperm-oocyte recognition and binding (Dravland and Meizel, 1982; Takano *et al*, 1993).

Once the spermatozoon has undergone the AR and passed through the zona pellucida and perivitelline space, the sperm head binds to the plasma membrane of the oocyte, probably via the plasma membrane overlying the equatorial region of the sperm head (Bedford and Cooper, 1978; Moore and Bedford, 1983). The two plasma membranes fuse, and the spermatozoon is finally incorporated into the egg cytoplasm (Yanagimachi, 1994). A number of molecules have been implicated in the binding of the spermatozoon to the vitelline membrane of the oocyte (for a review see Myles, 1993). One such protein is the PH-30 molecule of guinea pig spermatozoa, now called fertilin (Primakoff *et al*, 1987). This molecule has a disintegrin-like domain which may be involved in binding to an integrin molecule on the oocyte plasma membrane, leading to close association and ultimately fusion of the gametes and the subsequent activation of the oocyte (Blobel *et al*, 1992; Myles *et al*, 1994). Other putative sperm-egg binding/fusion molecules are galactotransferase in the mouse (Lopez and Sher, 1987); D.E. in the rat (Rochwerger, *et al* 1992), a 43kDa protein with some homology to CD46 (Okabe *et al*, 1990) and Clq (Fusi *et al*, 1991) all in the human.

2.2.5 Egg activation

Upon fusion with the spermatozoon, the dormant egg becomes activated and subsequently undergoes a series of cellular events that lead to cell division and differentiation, and result in the formation of a new individual. It has been suggested that the spermatozoon takes into the egg, a factor which initiates the activation (Nuccitelli, 1991), and the trigger for activation is thought to be a single transient increase in egg cytoplasmic free calcium (Ozil and Swann, 1992; Swann and Ozil, 1994). The signal transduction systems thought to be involved in egg activation include protein kinase C and G-protein-coupled pathways (Colonna et al, 1989; Moore et al, 1993), and inositol 1,4,5-triphosphate is also involved (Xu *et al*, 1994). The first signs of egg activation are the exocytosis of cortical granules and the resumption of meiosis (Yanagimachi, 1994). The egg completes meiosis, the egg pronucleus forms, and the sperm nucleus decondenses, transforming into the sperm pronucleus. The fully developed pronuclei congregate at the centre of the egg, their nuclear envelopes disintegrate, the chromosomes mingle, and then the first mitotic division ensues (Krishna and Generoso, 1977). This mingling of chromosomes from the male and female gametes is known as syngamy, and it marks the end point of fertilization and the beginning of embryonic development (Yanagimachi, 1994).

2.2.6 Block to polyspermy

The fertilization of the egg by more than one spermatozoon, i.e. polyspermy, is lethal for the embryo (Bedford and Rodger, 1983). There are two main mechanisms in mammals for the prevention of polyspermy. These two mechanisms are the cortical-granule-mediated zona reaction (ZR) (Austin and Braden, 1956; Bedford and Rodger, 1983; Cherr and Drucibella, 1990), and the plasma membrane block (PMB) (Menzies and Peters, 1985; Stewart-Savage and Bavister, 1988; Ng *et al*, 1990). The extent to which a given species relies on

each of the mechanisms varies. For example, in humans the ZR is the primary block to polyspermy, in the rabbit the PMB is the most important, whilst in the rat and the mouse, the ZR and the PMB are equally important (Yanagimachi, 1994).

The cortical reaction (CR) is an exocytotic event involving fusion of the oocyte plasma and cortical granule membranes, and is triggered by fertilization (Braden *et al*, 1954). The cortical granules are small, membrane-bound, lysozyme-like organelles that occupy the region of the oocyte cytoplasm just beneath the plasma membrane (Wassarman, 1987a). The fusion of the cortical granules with the vitelline membrane begins at the location of the sperm-egg fusion event, and is propagated, wave-like, across the fertilized oocyte. It is thought that a release of calcium from cytoplasmic stores, is responsible for the CR (Yanagimachi, 1994). As a result of the CR, the contents of the cortical granules are deposited in the perivitelline space. The enzymes from the cortical granules then modify then the zona pellucida, as manifested by a 'hardening' of the zona, and inactivation of sperm receptors in some species (Bleil and Wassarman, 1980; Wassarman, 1987a). However, in humans it appears that it is only hardening of the zona which occurs, without receptor inactivation (Bedford and Kim, 1993). The mechanisms responsible for zona hardening appear to involve the enzymatic degradation and modification of zona proteins. Some authors postulate that, similarly to the situation in the sea urchin, a cortical granule peroxidase induces cross-linking of tyrosine residues in the zona, rendering it hardened and impenetrable (Schmell and Gulyas, 1980; Gulyas and Schmell, 1980), but other authors favour zona hardening mechanisms dependent upon the actions of cortical proteases (Gwatkin *et al*, 1973; Shabanowitz and O'Rand, 1988) and glycosides (Wassarman, 1987a).

After fertilization, the ability of the oocyte plasma membrane to fuse with spermatozoa is dramatically reduced in most species, and this phenomenon constitutes the plasma membrane block to polyspermy

(Yanagimachi, 1994). Although, the mechanisms responsible for the PMB, in mammals, are not fully understood, it is thought not to be a direct consequence of the cortical reaction (Wolf *et al*, 1979).

2.2.7 Species-specificity of fertilization

There is some restriction on the ability of the gametes from two different species to participate in the events involved in fertilization and hence, form a new chimeric, individual. *In vitro*, the species-specificity of fertilization is controlled at the level of the zona pellucida and the oocyte plasma membrane (O'Rand, 1988). The species restriction is most pronounced at the zona pellucida, and sperm-zona interaction is considered to be quite species-specific, although spermatozoa and oocytes, from very closely related species, can interact and undergo the subsequent events of fertilization (O'Rand, 1988), e.g. human spermatozoa and gorilla oocytes (Lanzendorf *et al*, 1992).

Sperm interaction with the oocyte plasma membrane is much less species- specific than the sperm-zona interaction, with the spermatozoa and plasma membranes of very diverse animals being able to interact and fuse (Yanagimachi, 1994). The hamster oocyte, of all mammalian eggs so far studied, is the most able to allow fusion with spermatozoa of other species (Yanagimachi, 1984).

2.3 Reactive oxygen species (ROS)

Free radicals have been defined as molecular species that are capable of independent existence whilst containing one or more unpaired electrons in a given atomic or molecular orbital (Halliwell and Gutteridge, 1986). The possession of unpaired electrons is the feature which bestows upon these molecules their immense reactivity, and thus, their biological significance. The enhanced reactivity of these molecules is a result of the fact that more energy is required to maintain two separate species, each with an unpaired electron,

than to allow them to come together and share electrons such that a full molecular orbital is established, along with a covalent bond. Thus the reactivity of a free radical is inversely related to its stability. Oxygen radicals are very short lived species produced by the reduction of oxygen (Riley and Behrman, 1991). Examples of free radicals are the superoxide anion, $O_2^{-\cdot}$ and the hydroxyl radical, $OH^{\cdot}$. However, another very important reactive oxygen species, in biological terms, is hydrogen peroxide, H_2O_2. This compound does not possess any unpaired electrons, and is thus not strictly a free radical, but it is still an immensely reactive molecule. The addition of a single electron to molecular oxygen, leads to the formation of the superoxide anion, divalent reduction produces hydrogen peroxide, and trivalent reduction generates the hydroxyl radical. Throughout this thesis, this group of oxygen metabolites will be collectively referred to as reactive oxygen species (ROS).

Various cells contain enzymatic systems specifically for the generation of ROS. There exist at least four enzymatic mechanisms in mammalian cells, whose primary role is to generate free radicals, i.e. the NADPH-oxidase, nitric oxide synthase (NOS), lipoxygenase, and cyclooxygenase. The latter two enzymes are haem containing proteins which catalyze the direct reaction of various fatty acids with oxygen, yielding hydroperoxides and the hydroxyl radical in the case of lipoxygenase, and prostaglandins and a, as yet, unidentified oxygen radical in the case of cyclooxygenase (Halliwell and Gutteridge, 1986). Nitric oxide synthase is a flavoprotein enzyme, once described as neuronal diaphorase and endothelial relaxing factor (ERF) (Forstermann, 1994), and it catalyzes the formation of nitric oxide (a free radical species) from L-arginine (Fostermann, 1994; Nathan and Xie, 1994). NOS is present in various tissues and cells including, endothelial cells (Pollock *et al*, 1993) and neurones (Hope *et al*, 1991). Nitric oxide is a signalling molecule, with very important roles in various physiological processes

including arterial vasodilation (Lowenstein, 1994), through its capacity to activate guanylate cyclase.

However, the only enzyme described to date, primarily involved in generation of the superoxide anion is the NADPH oxidase, first described in phagocytic leucocytes (Rossi and Zatti, 1964), and this enzyme system is described briefly, below.

2.3.1 The NADPH oxidase

The NADPH oxidase was first described in phagocytic leucocytes, i.e. neutrophils, in 1964 (Rossi and Zatti, 1964), and has since received a great deal of attention (for reviews see Babior *et al*, 1987; Baggiolini and Wymann, 1990; Cross and Jones, 1991; Segal and Abo, 1993; Afanas'ev, 1994; Jones, 1994). The oxidase is an enzyme complex which generates the superoxide anion via the univalent reduction of oxygen, utilizing NADPH as electron donor. The oxidase is dormant in resting cells, but activated to a superoxide-generating state upon contact with the appropriate stimuli. Upon stimulation, it is the activity of the NADPH oxidase that is responsible for the so called 'respiratory burst' of the cell, which is necessary for the destruction of invading micro-organisms by phagocytes (Babior, 1978).

The NADPH oxidase complex itself, is composed of at least 5 components. The first of these characterized was the plasma membrane-bound, low potential cytochrome, cytochrome b_{558} (Segal and Jones, 1978). The cytochrome b_{558} is a heterodimer comprised of an α and ß subunit (Segal and Jones, 1978; Segal *et al*, 1979; Parkos *et al*, 1987), and is the terminal component of the electron transfer chain of the oxidase. Two other NADPH oxidase components are the cytosolic factors, $p47^{phox}$ and $p67^{phox}$. Both of these cytosolic components are translocated to the plasma membrane upon stimulation of the phagocyte, and this results in oxidase assembly and activation (Clark *et al*, 1990). The fifth, characterized oxidase component, is a

small GTP binding protein, $p21^{rac\ 1}$ (Knaus *et al*, 1992; Abo *et al*, 1991). This protein is once again involved in the assembly and activation of the oxidase, and associates with the cytochrome during the formation of the active NADPH oxidase complex (Quinn *et al*, 1989; Abo *et al*, 1991; Knaus *et al*, 1991).

Oxidase assembly and activation can be stimulated by a number of diverse, physiological and non-physiological stimuli, e.g. bacteria, opsonized particles, chemotactic peptides, PKC agonists, and ionophores (Babior *et al*, 1987; Morel *et al*, 1991; Segal and Abo, 1993), and stimulation results in the generation of the superoxide anion, which subsequently participates in the formation of a complex mixture of toxic oxidants, e.g. hydrogen peroxide, the hydroxyl radical, and hypochlorous acid (Babior *et al*, 1987). These oxidants are released into the phagocytic vacuole where they function to aid the phagocyte in the destruction of invading micro-organisms. Although the oxidants are manufactured purely for internal used, some inevitably leak into the surrounding tissues where they inflict various types of damage (Babior *et al*, 1987). This secondary, self-inflicted damage which can result from oxidase activation, is one of the factors which has lead to the great intensity of research into the oxidase. The NADPH-oxidase-associated damage experienced by the host, has now been shown to be a major factor in many pathological conditions and disease states (Gutteridge, 1993). Two of the most common pathological conditions, thought to be a direct consequence of phagocyte NADPH oxidase activity, are rheumatoid arthritis (McCord, 1974; Greenwald, 1986) and asthma (Barnes, 1990; Boelman and Blast, 1990). Recently, it has also been shown that the generation of the superoxide anion enhances the spread of HIV (human immunodeficiency virus) infection by cell-to-cell transmission (Kameoka *et al*, 1993), hence raising the possibility that ROS generation in the reproductive tract of the female, by spermatozoa and leucocytes, may, in fact, increase the likelihood of male-female transmission of the virus.

Another reason why so much work has been carried out, characterizing the NADPH oxidase, is that defects in this enzyme are responsible for the heritable condition of chronic granulomatous disease (CGD) (Tauber *et al*, 1983). Autosomal and X-linked mutations in the genes encoding the NADPH oxidase lead to this condition, which is characterized by the inability of the phagocytes of sufferers to mount a respiratory burst, and thus destroy invading pathogens. The disease is often fatal. However, the cells of sufferers of CGD have proved to be very useful tools for studying the NADPH oxidase, and their use has resulted in the elucidation of many of the signal transduction pathways regulating NADPH oxidase activity, as well as the characterization of it's component parts (e.g. Royer-Pokora *et al*, 1986; Teahan *et al*, 1987).

In recent years it has become increasingly apparent that phagocytic leucocytes are not the only cell types capable of generating ROS by NADPH oxidase-like mechanisms. Other such cells include glomerular mesangial cells (Radeke *et al*, 1991), fibroblasts (Meier *et al*, 1991), endothelial cells (Sundquist, 1991), Epstein-Barr transformed B-lymphocytes (Hancock *et al*, 1991), thyroid cells (D□me *et al*, 1985), adipocytes (Krieger-Brauer and Kather, 1992) and osteoclasts (Steinbeck *et al*, 1994), to name only a few, and finally, of course, the spermatozoon (Alvarez *et al*, 1987; Aitken and Clarkson 1987a and b). The work carried out on the NADPH oxidase of phagocytic leucocytes has provided a very useful framework upon which to conduct research into the ROS generating abilities of other, non-phagocytic cells, and to deduce their physiological functions. In fact, a picture is beginning to emerge showing that ROS have fundamental, signalling roles in the normal physiology of many cells and the tissues surrounding them (Saran and Bors, 1989; Schreck and BŠuerle, 1991; Satriano *et al*, 1993); it is now thought probable that ROS are ubiquitous modulators of cell function.

2.3.2 Lipid peroxidation

One of the main actions of reactive oxygen species is the induction of lipid peroxidation, and this can have profound effects on the organization and function of biological membranes (Halliwell and Gutteridge, 1986). The susceptibility of biological membranes to peroxidation is largely due to the presence of polyunsaturated fatty acids. Polyunsaturated fatty acids are particularly prone to oxidative attack because the presence of a double bond weakens the C-H bonds on the adjacent carbon atoms, thereby facilitating the hydrogen abstraction process that initiates the lipid peroxidation cascade (Halliwell and Gutteridge, 1986). Neither the superoxide anion nor hydrogen peroxide are energetic enough to initiate lipid peroxidation directly, but in the presence of catalytic amounts of transition metals, such as iron or copper, they react (the **'Haber-Weiss Reaction'**) and the hydroxyl radical is formed (Di Guiseppi and Fridovich, 1980; Halliwell and Gutteridge, 1992; Lichochev and Fridovich, 1994). This molecule is a direct, and powerful initiator of lipid peroxidation (Halliwell and Gutteridge, 1986).

(1) $$O_2^{\cdot-} + H_2O_2 \longrightarrow \underset{\text{hydroxyl radical}}{{}^{\cdot}OH} + \underset{\text{hydroxyl ion}}{{}^{-}OH} + O_2$$

The hydroxyl radical initiates lipid peroxidation by abstracting a hydrogen atom from a lipid $-CH_2-$ group, creating a lipid radical ($-{}^{\cdot}CH-$), which then undergoes a molecular rearrangement to form a conjugated diene. The latter will then react with oxygen to form a peroxyl radical, ROO$^{\cdot}$, which stabilizes by abstracting a hydrogen atom from an adjacent lipid to form a lipid hydroperoxide, ROOH. Thus, a lipid radical is created out of the second lipid, and a chain reaction is initiated, which propagates the damage throughout the membrane, unless a chain-breaking anti-oxidant intercedes, such as α-tocopherol (vitamin E). A schematic representation of lipid peroxidation is presented in figure 2.3.

From this brief description of lipid peroxidation, it can be appreciated that ROS can have a substantial impact on biological membranes, and as membrane function plays a very important role in the events that define the fertilizing potential of the human spermatozoon, it is obvious that ROS generation by these cells has the potential to alter profoundly their functional capability. In the following sections we shall examine the interface between free radical chemistry and gamete biology, and consider the nature of the interaction between ROS and human spermatozoa.

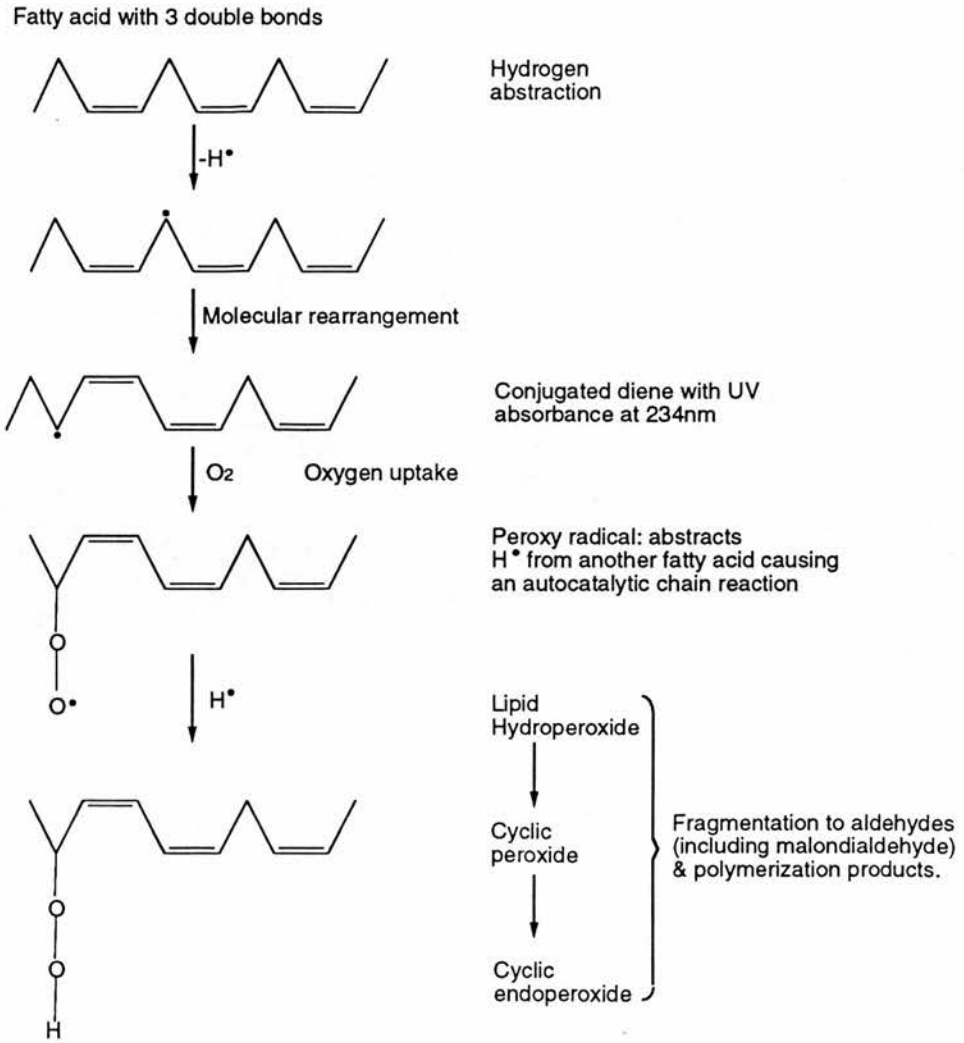

Figure 2.3 Schematic representation of the initiation and propagation reactions of lipid peroxidation The peroxidation of a fatty acid with three conjugated double bonds is shown. For further details see text.

2.3.3 Reactive oxygen species and spermatozoa

The first indication that mammalian spermatozoa generated ROS came from John Macleod who, in 1943, observed that the addition of catalase, a scavenger of hydrogen peroxide, to human spermatozoa protected them from the motility loss observed when they were incubated under high oxygen tension (MacLeod, 1943). From these results, MacLeod (1943) postulated that the spermatozoa themselves generated hydrogen peroxide, via the two electron reduction of molecular oxygen. Three years later, Tosic and Walton published a paper providing the first direct evidence that mammalian spermatozoa have the capacity to generate ROS, specifically hydrogen peroxide (Tosic and Walton, 1946). This work was carried out on bull spermatozoa, and subsequent studies have shown that the spermatozoa of the mouse (Alvarez and Storey, 1984), rabbit (Holland *et al*, 1982), rat (Kumar *et al*, 1990), and hamster (Bize and Sharpe, 1990), are all capable of ROS generation. The first direct evidence for ROS generation by human spermatozoa was published in 1987, independently by Alvarez *et al* (1987) and Aitken and Clarkson (1987a and b). The cellular mechanisms responsible for the generation of ROS by spermatozoa remain to be fully elucidated, although it is already apparent that some variation may exist between species. For example, work on bovine spermatozoa has suggested that ROS are generated via the oxidative deamination of aromatic amino acids (Tosic and Walton, 1950). In contrast, the reactive oxygen species generated by rabbit spermatozoa appear to derive from the leakage of electrons from the mitochondrial electron transport chain (Killian *et al*, 1985). Neither of these mechanisms appears to apply to the generation of ROS by human spermatozoa since neither aromatic amino acids nor mitochondrial inhibitors appear to influence the rate at which reactive oxygen species are produced by these cells (Aitken and Clarkson, 1987a and b; Aitken and Ford, 1988). In the case of human spermatozoa the evidence points to the existence of a mechanism that is similar, though not identical, to that of phagocytic

leucocytes, i.e. via a NADPH-oxidase complex. The stimulation of superoxide anion generation by detergent-solubilized human spermatozoa, in response to the administration of exogenous NADPH (Aitken and Ford, 1988), suggests that this coenzyme is the probable source of electrons for the reduction of oxygen by these cells. The superoxide generated then dismutates, under the influence of superoxide dismutase (SOD), to hydrogen peroxide (Mennella and Jones, 1980; Aitken and Clarkson, 1987a and b; Alvarez *et al*, 1987; Aitken and Ford, 1988; de Lamirande and Gagnon, 1992a).

(2) $$NADPH + 2O_2 \longrightarrow 2O_2^{-\cdot} + NADP^+ + H^+$$

(3) $$2O_2^{-\cdot} + 2H^+ \xrightarrow[SOD]{} O_2 + H_2O_2$$

Recently, it has also been shown that the generation of ROS by human spermatozoa, and the activity of glucose-6-phosphate dehydrogenase, the enzyme that controls the rate of glucose through the hexose monophosphate shunt (the enzyme system responsible for the supply of NADPH) are positively correlated (Aitken *et al* 1994b), suggesting that ROS generation by human spermatozoa is indeed dependent on the availability of NADPH, like their phagocytic counterparts (Cross *et al*, 1984).

Further parallels between the ROS generating system in spermatozoa and the NADPH-oxidase of phagocytic leucocytes can be drawn from the fact that the stimulation of protein kinase C, by phorbol esters induces ROS generation in both cell types (Repine *et al*, 1974; Wymann *et al*, 1987; Rossi *et al*, 1986; Dieter, 1992; Curnutte *et al*, 1994; Kovalski *et al*, 1992; Aitken and West, 1990; Iwasaki and Gagnon, 1992; Robinson *et al*, 1985; Aitken and Buckingham, 1992; Aitken *et al*, 1992a and b; Krausz *et al*, 1992; Aitken *et al*, 1993a; Krausz *et al*, 1994) as does the divalent cation ionophore A23187 (Aitken and Clarkson, 1987a and b; Becker *et al*, 1979) Despite these similarities in second messenger regulation, no direct evidence has yet been obtained indicating that the

mechanisms via which phagocytic leucocytes and spermatozoa generate ROS are biochemically identical, and the resolution of this situation is one of the main aims of this thesis.

2.3.4 Reactive oxygen species and sperm peroxidative damage

It can be appreciated that ROS generation is likely to have a profound effect on the physiology of human spermatozoa. It has been shown that these molecules can have a pathological effect on sperm function, through the initiation of excessive lipid peroxidation in the sperm plasma membrane (Jones *et al*, 1979; Alvarez *et al*, 1987; Aitken and Clarkson 1987a and b; Aitken *et al*, 1989a; Aitken *et al*, 1993b). As indicated above, human spermatozoa are susceptible to such damage because they have plasma membranes that are enriched with unsaturated fatty acids, and also they possess a relative lack of the cytoplasmic enzymes that usually protect against oxidative stress in somatic cell types. Also, such enzymes are only present in the mid-piece of the spermatozoon, thus affording no protection to the plasma membrane over the head and tail regions of the cell (Jones *et al*, 1979; Alvarez *et al*, 1987; Aitken and Clarkson, 1987a). To some extent the lack of cytoplasmic defensive enzymes is compensated for by the antioxidant properties of seminal plasma, that is well endowed with a variety of factors that are designed to protect the spermatozoa against oxidative stress (Jones *et al*, 1979; Zini *et al*, 1993). These factors include small molecular weight scavengers such as ascorbic and uric acid, as well as antioxidant enzymes such as superoxide dismutase and catalase. Thus, as long as spermatozoa are suspended in seminal plasma they have some protection against oxidative stress. This could be an important factor in protecting spermatozoa from the ROS generated by any neutrophils that are released into the semen at the moment of ejaculation from the secondary sexual glands (Wolff and Anderson, 1988), as such cells do contribute to the ROS generated in semen (D'Agata, 1990; Aitken and West, 1990). Evidence for such a

protective effect is suggested by the fact that leucocyte contamination in semen does not have any deleterious effect on subsequent sperm function (Tomlinson *et al*, 1993; Aitken *et al*, 1994a). Moreover, this is the case even for levels of contamination classed by the World Health Organization as abnormal, i.e. > $1x10^6$/ml (WHO, 1992). In contrast, as soon as spermatozoa are released from the protection afforded by seminal plasma, as when they colonize the cervix, or are washed in preparation for *in vitro* fertilization therapy, they become extremely sensitive to oxidative stress, including that contributed by contaminating leucocytes (Aitken and Clarkson, 1988; Aitken and West, 1990; Kolvalski *et al*, 1992; Aitken *et al*, 1992a; Kessopoulou *et al*, 1992; Aitken *et al*, 1994a, Plante *et al*, 1994). The ROS generated by, even a low concentration of, contaminating leucocytes will seriously impair the capacity of washed sperm preparations for fertilization, although, a significant improvement in fertilization rates can be achieved by selectively removing the leucocyte population using magnetic beads coated in a monoclonal antibody against the common leucocyte antigen (Krausz *et al*, 1992; Aitken *et al*, 1994a; Aitken *et al*, 1995a) or by adding antioxidants to the incubation mixture (Aitken and Clarkson, 1988; Aitken *et al*, 1989a; Kobayashi *et al*, 1991; Kovalski *et al*, 1992).

However, such strategies for relieving oxidative stress are redundant if the source of the ROS is the spermatozoa themselves, rather than contaminating leucocytes. In men with abnormal semen profiles, particularly those classified as oligozoospermic, it is the spermatozoa that appear to constitute the major source of ROS (Aitken and Clarkson 1987a; Aitken and Clarkson, 1987b; Aitken *et al*, 1989b; Aitken and West, 1990; Aitken *et al*, 1992a; Aitken, 1994a; Aitken *et al*, 1995a). In such cases, sperm function may be so damaged by the time of ejaculation that it is not possible to reverse the damage. Analysis of the lipo-peroxidative status of human spermatozoa suggests a very close relationship between the accumulation of lipid peroxides in the sperm plasma membrane and the inhibition of both motility and sperm-

oocyte fusion (Jones *et al*, 1979; Alvarez *et al*, 1987; Aitken *et al*, 1989a; de Lamirande and Gagnon, 1992a and b; Aitken *et al*, 1993b).

In conclusion, it does appear that high levels of ROS generation by human spermatozoa is associated with some forms of male infertility(Aitken 1994 a and b; Aitken *et al*, 1995a). However, it is not certain what causes some sperm populations to generate elevated levels of ROS compared to their fertile counterparts. One burgeoning hypothesis is that the capacity to generate ROS is linked to the retention of excess residual cytoplasm during the final stages of spermatogenesis and hence, the enhanced presence of certain cytoplasmic enzyme (Aitken *et al*, 1994b). Thus, it has been shown that the levels of peroxidative damage sustained by spermatozoa are, indeed correlated with mid piece defects (Rao *et al*, 1989). Such defects are often characterized by the retention of excessive amounts of cytoplasm, and high levels of several cytoplasmic enzymes including lactate dehydrogenase (Casano et al, 1991; Gavella and Lipovac, 1992; Gavella and Lipovac, 1993; Velasco *et al*, 1993; Orlando *et al*, 1994), diaphorase (Gavella and Lipovac, 1992; Gavella and Lipovac, 1993), creatine phosphokinase (CPK) and glucose-6-phosphate dehydrogenase (G6PDH) (Huszar *et al*, 1988; Huszar *et al*, 1990; Aitken *et al* 1994b). The majority of these enzymes are probably just markers of the cytoplasmic space, as opposed to being directly linked to the ability of ROS generation. However, the levels of G6PDH do show a direct correlation with levels of ROS generation (Aitken *et al*, 1994b), presumably through the capacity of the enzyme to supply the putative ROS generating oxidase complex with NADPH. Thus, morphological analysis of mid piece area and/or biochemical evaluation of cytoplasmic enzyme content (e.g. G6PDH) may prove to be a useful diagnostic tool in evaluating sperm fertilizing ability.

2.3.5 Reactive oxygen species and sperm physiology

Although it is well documented that excessive ROS generation by spermatozoa can lead to a pathological loss of sperm function (see above), it seems unlikely that these cells would have retained such a potentially dangerous biochemical mechanism if ROS did not play a role of some importance in the normal process of fertilization. In support of this contention, evidence has recently been obtained to suggest that ROS are involved in both sperm capacitation and in the induction of the acrosome reaction (Bize and Sharpe, 1990; Bize *et al*, 1991; de Lamirande and Gagnon, 1993a, b and c; de Lamirande and Gagnon, 1995). Superoxide, generated *in vitro*, with the xanthine/xanthine oxidase system, has been shown to stimulate the hyperactivation of human spermatozoa, providing catalase is present in the incubation mixture to scavenge any hydrogen peroxide present (de Lamirande and Gagnon, 1993a and b). The specificity of this hyperactivation-inducing effect was indicated by its susceptibility to inhibition by superoxide dismutase. Moreover, de Lamirande and Gagnon have also shown that the ability of complex biological fluids to induce hyperactivation is inversely related to their superoxide dismutase content (de Lamirande and Gagnon, 1993c.) It addition, it has been shown that human spermatozoa show enhanced levels of ROS generation when cultured under conditions that promote capacitation, and that substances capable of promoting this process, e.g. follicular fluid components and foetal cord serum, stimulate ROS generation by human spermatozoa (de Lamirande and Gagnon, 1995). Moreover, it has been shown that superoxide dismutase was capable of inhibiting capacitation by human spermatozoa (de Lamirande and Gagnon, 1993a and b; de Lamirande and Gagnon, 1995).

Interestingly, although it is the superoxide anion which appears to stimulate capacitation in human spermatozoa, in the hamster it appears that hydrogen peroxide is the ROS involved (Bize and Sharpe, 1990; Bize *et al*, 1991), and that this molecule actually stimulates the acrosome reaction in the

spermatozoa of this species. The *in vitro* system employed to generate this powerful oxidant contained a combination of glucose and glucose oxidase (Bize and Sharpe, 1990; Bize *et al*, 1991). Exposure of hamster spermatozoa to reactive oxygen species generated in this way, resulted in the stimulation of the acrosome reaction. In this instance, the biological activity of the reactive oxygen species generated by glucose oxidase could be blocked by the inclusion of catalase in the incubation medium, to scavenge the hydrogen peroxide generated by this enzyme. Such results suggest that the generation and release of hydrogen peroxide by these cells is an important element in their activation.

In general a species specificity for the ROS directly involved in modulating sperm function seems unlikely, and it is probable that the two ROS molecules mentioned above fulfil different roles, i.e. the superoxide anion is involved in the acquisition of a capacitated state, whilst hydrogen peroxide is actually involved in the induction of the acrosome reaction.

Further evidence supporting a physiological role of ROS generation in sperm function is that sub-lethal levels of ROS-induced lipid peroxidation, incurred by spermatozoa, result in enhanced sperm-zona interaction (Aitken *et al*, 1989a). More recently, it has been shown that phosphorylation of sperm tyrosine containing proteins, thought to be involved in sperm-zona interaction and induction of the acrosome reaction (Naz and Ahmad, 1991; Leyton *et al*, 1992), is profoundly influenced by the redox status of the spermatozoa, and that addition of exogenous hydrogen peroxide to spermatozoa increases levels of tyrosine phosphorylation, with a concomitant increase in the rates of acrosome reaction and sperm-oocyte fusion (Aitken *et al*, 1995b). Stimulation of exogenous ROS generation, was shown to exert a similar effect, which could be inhibited by the addition of catalase to the sperm suspensions, indicating that hydrogen peroxide was the ROS molecule involved (Aitken *et al*, 1995b).

2.4 Conclusions

In conclusion, mammalian spermatozoa express the ability to generate ROS, whilst lacking the vigorous defence mechanisms necessary to protect themselves against the potentially deleterious effects of these highly reactive molecules. With this in mind it seems that ROS must play an important role in the physiology of spermatozoa, and the complicated series of changes they must undergo before they can effect fertilization. However, a growing body of evidence links excessive ROS generation by spermatozoa with sperm dysfunction and infertility. Thus, these cells are clearly in the precarious situation of having to ensure the delicately balanced generation of these potentially harmful, but probably biologically important, modulators of cell function. It can be appreciated that an understanding of the cellular mechanisms which underlie the genesis of ROS by spermatozoa, would be extremely useful in both understanding fertilization as a whole, and treating peroxidative damage associated male sub-fertility. With this in mind, the aims of this thesis are to attempt to dissect out the cellular mechanisms and molecules, involved in ROS generation by mammalian spermatozoa.

Chapter 3

General materials and methods

The methods outlined below are the general experimental protocols and procedures that relate to more than one chapter of this thesis. Methods that are only relevant to individual chapters, are described in the appropriate section.

3.1 Semen Donation

Human semen samples were obtained from a large panel of volunteer donors. All of the donors had been clinically examined and shown to be free of any detectable pathology, including hepatitis and HIV infection. The semen samples were produced by masturbation and collected in sterile, plastic containers (Sterilin, Bibby Sterilin Ltd., Stone, England) and transported immediately to the laboratory for subsequent analyses and experimentation. Each semen sample was allowed to liquefy for at least 30 minutes, before conducting a routine semen analysis, to ensure that it could be classified as exhibiting a normozoospermic semen profile according to the criteria laid down by the World Health Organisation (WHO, 1992), i.e. volume > 2ml, concentration > 20 x 10^6/ml, motility > 50% with forward progressive movement, morphology > 30% normal.

3.2 Isolation and Fractionation of Spermatozoa

After the routine semen analyses had been performed, the semen samples were isolated from the seminal plasma and fractionated on a two step Percoll® gradient, as described by Aitken and Clarkson (1988), with a few minor modifications, as follows. The isotonic Percoll used in the gradient was created by supplementing 90ml of Percoll (Pharmacia, Pharmacia LKB,

Knowlhill, Milton Keynes, UK) with 10ml of 10X concentrated medium 199 (Flow Laboratories, Irvine, Scotland), 1.5ml 20% albuminar (human serum albumin solution; Immuno Ltd., Sevenoaks, UK), 3mg sodium pyruvate, 0.37ml sodium lactate (both Sigma Chemical Company, Poole, Dorset, UK), and 200mg sodium hydrogen carbonate (BDH Chemicals, Poole, Dorset, UK). This isotonic Percoll preparation was designated 100% Percoll (Lessley and Garner, 1983), and a 50% preparation was made by diluting it 1:1 with HEPES (Flow Laboratories) buffered medium BWW (Biggers *et al*, 1971), supplemented with 0.3% albuminar. The 2 step, discontinuous gradient used for routine sperm preparation consisted of 3ml of 100% Percoll placed in the bottom of a sterile 15ml conical bottomed, plastic tube (Falcon, Becton Dickinson and Company, Plymouth, UK), overlaid with 3ml of the 50% Percoll. Care was taken to ensure a clear interface existed between the 100% and 50% Percoll layers. Next, 1-3ml of liquefied semen was carefully laid on the top of each gradient and the whole preparation was centrifuged for 20 minutes at 500*g* in a 500-E Wifuge swing out rotor centrifuge (Scotlab, Coatbridge, Scotland). After centrifugation, the seminal plasma remaining above the 50% Percoll, was discarded. The cell populations at the 100%-50% interface and at the base of the 100% Percoll were then collected and designated the '50% and '100%' fractions respectively. The separate sperm fractions were subsequently washed with approximately 10ml of BWW, by centrifuging at 500*g* for 5 minutes. The washed cells were finally resuspended in fresh BWW to give a concentration of $20x10^6$ spermatozoa/ml, the cell concentration being determined using an improved Neubauer haemocytometer (BDH Chemicals). The cells were maintained at 37°C in a dry heating block until use. All subsequent experiments were carried out with the spermatozoa in BWW, at a cell concentration of 20×10^6 spermatozoa/ml, unless stated otherwise.

Characterisation of Cell Fractions

100% Fraction

This fraction contained the cells that are classified as the 'high quality' spermatozoa of the ejaculate. These cells were characterised by being of high density, possessing good motility and morphology, and by performing very well in biological assays of sperm function, e.g. induction of the acrosome reaction, zona pellucida-free hamster egg penetration tests (HEPT), and human zona pellucida binding assays (Aitken and Clarkson, 1988.). This fraction of cells was also characterised by having very few contaminating leucocytes and immature germ cells present.

50% Fraction

This fraction contained the poorest quality spermatozoa, exhibiting low density, poor motility and abnormal morphology. These cell suspensions were also characterised by their poor performance in assays of biological function (Aitken and Clarkson, 1988), and were frequently contaminated with leucocytes and immature germ cells.

3.3 Detection of Reactive Oxygen Species Generation

ROS generation was mainly monitored via luminometry employing the chemiluminescence probes lucigenin and luminol. Lucigenin specifically detects the superoxide anion, whereas the more sensitive probe luminol, when in conjunction with horseradish peroxidase (HRP), detects mainly hydrogen peroxide (Aitken *et al*, 1992b). The principles underlying the assays of chemiluminescence were explained in an earlier chapter of this thesis. The protocols followed for using each probe were as follows. Briefly, the methods employed were very similar to those described by Aitken et al (1992b) with a only a few minor modifications. Chemiluminescence was monitored using a six chamber, Berthold 9505 C luminometer (Berthold, Wilbad, Germany),

maintained at a constant temperature of 37° C, linked to a Hewlett-Packard IBM compatible PC (Hewlett-Packard Company, Camas, WA, USA). Chemiluminescence was detected by 6 electronically compensated photomultipliers, associated with the six luminometer chambers, and expressed graphically, via a PC running on Berthold 9505 software package, as chemiluminescent counts per minute. For both of the probes used, 400µl of a suspension of spermatozoa, at a concentration of 10×10^6 per ml, were placed in a 3ml plastic, flat-bottomed luminometer tube (Denlley Instruments, Luckham Division, Billinghurst, England). When luminol was used, the spermatozoa were suspended in normal BWW, but when lucigenin was used, the spermatozoa were firstly spun down at 500*g* for 5 minutes, and then resuspended in BWW lacking albuminar, but supplemented with 1mg/ml polyvinyl alcohol (PVA; Sigma). Next, 4µl of a 25mM stock solution in dimethylsulphoxide (DMSO, BDH) of either luminol or lucigenin (both Sigma), was added to the cell suspension, resulting in a final working probe concentration of 250µM. The stock solutions of the probes were stored protected from light and refrigerated at 4° C, when not in use. When luminol was used, 8µl of horseradish peroxidase, at a concentration of 2mg/ml (HRP, Type VI, 288U/mg, Sigma), in phosphate-buffered saline (PBS, Flow Laboratories), was also added to the cell suspension, resulting in a final HRP working concentration of 11.52U/ml. The HRP stock solution was made up fresh each day. The tubes containing the cells and probe were then transferred to the luminometer as quickly as possible to prevent any light induced inactivation of the probes. Probe-dependent chemiluminescence was then monitored in the luminometer, and once a steady basal signal had been achieved, usually after around ten minutes, chemical reagents and any other factors to be investigated for their effects are ROS generation, were added to the cell suspensions at the appropriate concentrations. Measurement of

chemiluminescence was briefly suspended whilst any additions to the tubes were made, and resumed immediately after addition.

Analyses of the results from these assays were either carried out via the comparison of whole luminometer run traces, or via the comparison of the integrated number of counts detected over a specified time window of the luminometer run.

3.4 Assessment and Removal of Contaminating Leucocytes.

Assessment of Leucocyte Contamination

As can be easily appreciated, the presence of leucocytes, prolific generators of ROS in their own right, in a suspension of spermatozoa is of paramount concern when investigating ROS generation by spermatozoa. Conclusive evidence is required to establish that it is the spermatozoa that are generating any ROS detected, and not any infiltrating leucocytes. The presence of leucocytes in the suspensions of spermatozoa was evaluated in two ways. Firstly, a crude microscopic visual assessment was carried out, this involved simply counting the number of 'round' cells present in the suspensions. Secondly, a very sensitive leucocyte provocation test, involving the chemotactic peptide N-formyl-methionyleucylphenylalanine (FMLP), was employed to biochemically determine the absence or presence of leucocytes in the cell suspensions. The provocation test was carried out as follows, following the method described by Krausz *et al* (1992). Briefly, 400µl of a suspension of spermatozoa at a concentration of 10×10^6/ml were placed in a 3ml luminometer tube. ROS generation by the cell suspension in response to FMLP was then measured by HRP-enhanced luminol-dependent chemiluminescence as described above. Once a steady, basal chemiluminescent signal was achieved, the suspension was challenged with 2µl of a stock solution of 10mM FMLP (Sigma) in DMSO, resulting in a final

working concentration of 50µM FMLP. Any resulting chemiluminescence due to ROS generation was subsequently monitored. A FMLP-induced increase in the chemiluminescence, no matter how slight, was taken to indicate the presence of leucocyte contamination. A graph showing a typical result from an FMLP provocation test is shown in figure 3.1.

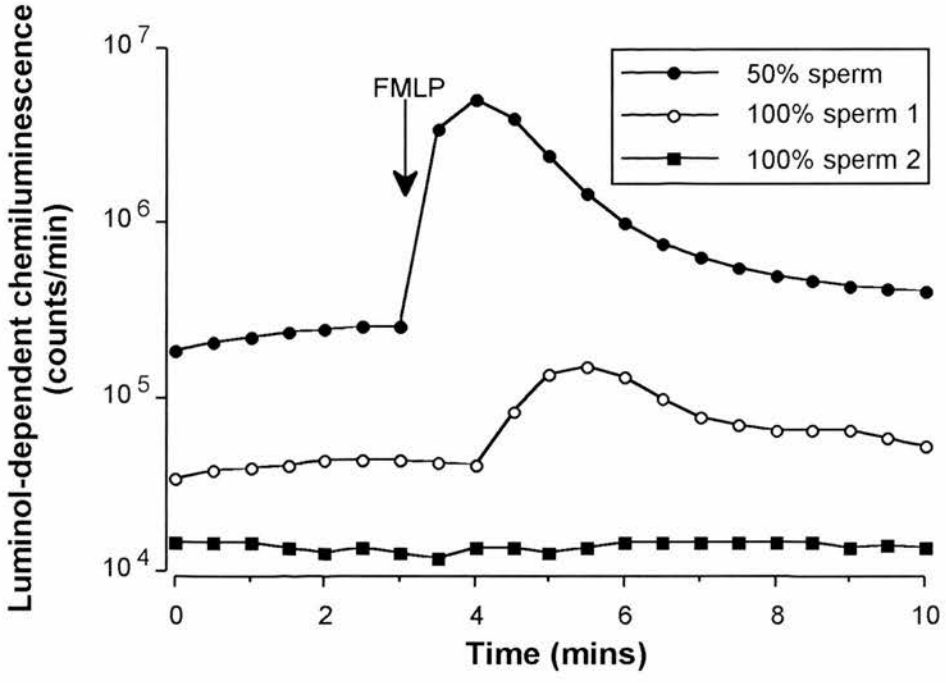

Figure 3.1 FMLP provocation test on human sperm suspensions. FMLP (50µM) was added to sperm suspensions and the resulting HRP-enhanced, luminol-dependent chemiluminescence monitored. In this example, the 50% sperm suspension and the 100% sperm (1) suspension both responded positively to the test, and are therefore, leucocyte contaminated. The 100% sperm (2) suspension did not respond positively, and is therefore, leucocyte free.

Leucocyte Removal

Cell suspensions that responded positively to the FMLP challenge test were either not used in assays of ROS generation, or they were subjected to the following protocol in order to remove all the contaminating leucocytes present. Total leucocyte removal was accomplished via a magnetic cell separation technique, first described by Krausz et al (1992). This technique

involved the use of magnetic M-450 Dynabeads® coated with sheep anti-mouse immunoglobulin G (Dynal AS, Oslo, Norway) which were subsequently labelled with a monoclonal antibody raised against the common leucocyte antigen CD45 (Scottish Antibody Production Unit, Carluke, Scotland). 1ml of sperm suspension was placed in a 3ml plastic luminometer tube, to which was added 40µl of the antibody labelled Dynabeads (suspended in PBS). This preparation was then incubated on a slowly rotating wheel (Stuart Scientific, Redhill, England) for 30-60 minutes, at room temperature. After this time, the Dynabeads were removed by the means of a simple magnet. The resulting cell suspension was then re-challenged with FMLP. The leucocyte removal procedure was repeated until the suspension of spermatozoa was proved to be free of any contaminating leucocytes.

3.5 Protein Solubilization

Two main methods of protein solubilization were employed during the course of this thesis both based on Tris-detergent protein extraction buffers. Briefly, prior to protein solubilization, cells were washed three times with a Tris based washing buffer comprising of 62.2mM Tris (electrophoresis grade, Sigma) titrated to pH 6.8 with hydrochloric acid (BDH Chemicals). The washing procedure involved centrifugation at 500*g* for 5 minutes for each wash cycle. The resulting washed cell pellet was then incubated with the appropriate solubilization buffer according to the following protocols.

SDS Extraction

The cells were incubated with an appropriate volume of Tris-SDS solubilization buffer (Tris-HCl, 62.5mM, pH 6.8; glycerol 10%; 2% SDS (sodium dodecyl sulphate), 22µg/ml aprotinin (all Sigma); AEBSF, 1mM (Calbiochem, Novabiochem, Nottingham, UK). This mixture was then incubated at room temperature for 45 minutes, vortexing occasionally. After

this time, the resulting preparation was centrifuged at approximately 10,000g for 20 minutes in a microcentrifuge (Howie). The supernatant, containing the SDS solubilized protein, was then removed and stored at 4° C until use.

OTG Extraction

The cells were incubated with an appropriate volume of Tris-OTG buffer (Tris-HCl, 62.5mM, pH 6.8; glycerol, 10%; 22µg/ml aprotinin (all Sigma); 4-(2-aminoethyl)-benzenesulphonylfluoride (AEBSF) 1mM; OTG (n-octyl-ß-D-thioglucoside) 1.1% (both Calbiochem), on ice, for 30 minutes, vortexing occasionally. After this time the preparation was centrifuged at approximately 10,000*g* for 30 minutes in a microcentrifuge, maintained at 4° C. The supernatant, containing the OTG solubilized protein, was subsequently removed and frozen at -20° C until required.

3.6 Estimation of Protein Concentration

Estimation of the protein content of samples was carried out using a bicinchoninic acid (BCA) protein estimation kit from Pierce (Pierce), which is based upon the method described by Smith et al (1985). Protein estimation was carried out following the protocol that came with the kit. This involved the preparation of a series of protein standards against which the unknown samples could be compared. The standards comprised of a range of concentrations of bovine serum albumin (BSA, Sigma) in PBS, ranging from 5µg/ml up to 1.2mg/ml protein. Each standard and unknown was set up in duplicate and involved the incubation of 50µl of standard or unknown with 1ml of working reagent. The working solution was prepared immediately before use, by mixing the BCA detection reagent with a 4% copper sulphate solution at a ratio of 50:1. Blanks were produced by substituting PBS and the diluent of the unknown protein samples for the actual protein samples. Prepared samples (the standards, unknowns, and blanks, i.e. PBS and the

diluents if the unknowns) were then incubated at 37°C for 30 minutes (normal protocol) or for 30 minutes at 60°C (enhanced protocol), the 60°C incubation resulting in a greater sensitivity and accuracy at low protein concentrations. The developed samples were then vortexed and transferred to disposable, plastic cuvettes (BDH Chemicals) and their absorbance, at 562nm, measured using a Shimadzu UV-2101 UV-VIS spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA). The spectrophotometer was linked to an Ambra Sprinta 486 IBM compatible PC (Scotbyte, Livingston, Scotland UK) that ran Shimadzu software. This software was used to directly plot the standard absorbance curve and then calculate the protein concentrations of the unknowns from the standard curve generated. The means and standard errors of the data were also calculated.

3.7 Protein electrophoresis

Solubilized protein preparations were subjected to one dimensional sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using a Protean electrophoresis unit (Bio Rad, Hemel Hempstead, Herts.,, England). The resolving gels were cast the day prior to the electrophoretic run to allow complete polymerization of the gel, and were composed of either 5%, 7.5%, or 10% acrylamide in Tris buffer(Tris-HCl, 0.25M, pH 8.8). The cast gels were stored at 4°C overnight, overlaid with Tris buffer, 0.25M, pH 8.8. The stacking gel was poured on the day of the electrophoresis run and was composed of 3.6% acrylamide in Tris buffer, pH 6.8. In both cases, polymerization of the acrylamide gels was catalyzed with 0.005% N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.0005% ammonium persulphate, both of which were purchased from Bio Rad. Immediately after pouring the stacking gel a sample application comb was inserted into the gel, so to create the wells for the protein samples; 10, 15, and 20 well combs were used. Prior to electrophoresis, protein samples were

denatured under non-reducing or reducing conditions. This involved the mixing of the samples 1:1 with sample buffer, comprising of 2% SDS, 10% sucrose (w/v), 0.001% bromophenol blue (w/v) in 187mM Tris, pH 6.8. If the samples were to be electrophoresed under reducing conditions the sample buffer also contained 0.05% (v/v) 2-mercaptoethanol. The protein preparations were then heated to 100°C for five minutes before being applied to the gel. Once the samples had been applied to the gel, along with the appropriate molecular weight standards, electrophoresis was carried out in a Tris-glycine electrode buffer (Tris, 25mM; glycine, 192mM) containing 0.1% SDS, at a constant current of 30mA/gel through the stacking gel and at 20mA/gel through the resolving gel. Electrophoresis through the resolving gel was allowed to proceed until the dye front had reached approximately 1cm. from the bottom of the gel. This generally took approximately 3 hours. After electrophoresis, the gels were removed from the apparatus and either stained, or blotted onto nitrocellulose membranes in order to undergo Western blotting analyses.

3.8 Coomassie Brilliant Blue staining

Gels for Coomassie blue staining were submerged in a staining solution containing 0.25% Coomassie Brilliant Blue R (Sigma) in 40% methanol (BDH), 10% acetic acid(BDH). Gels were incubated in this solution, on a rocking table, for a few hours or overnight, at room temperature. De-staining was carried out by incubating the stained gel in 20% methanol, 5% acetic acid, containing a spatula of anion exchanger resin (DEAE Sephadex, A25, Sigma). Addition of the anion exchanger speeds up the de-staining process. The de-staining solution was changed 2-3 of times, when necessary, and de-staining continued until the gels background was completely destained, and the protein bands visible. Gels were stored in clean de-stain solution until a permanent photographic record could be made.

3.9 Silver staining

Silver staining was performed using a commercially available kit (Bio Rad, Hemel Hempstead, Herts, UK), according to the manufacturers instructions. All solutions were made up, immediately prior to use, using distilled-deionized water, and all incubations were performed at room temperature on a rocking platform. Briefly, the gels were fixed in 40% methanol (BDH), 10% acetic acid (BDH) for 30 minutes and then incubated in 2 changes of 10% ethanol (BDH), 5% acetic acid, for 15 minutes. Gels were subsequently incubated in the oxidizer solution (20ml oxidizer concentrate, 180ml water) for 5 minutes and then subjected to three, 5 minute washes in water. The gels were incubated in the silver reagent solution (20ml silver reagent, 180ml water) for 20 minutes, and then washed for 1 minute in water. The gels were finally developed in developing solution (16g developer, 500ml water) for 5 minutes, the developing solution replaced and the developing continued until the desired level of staining was observed: staining was stopped by submerging the gels in 5% acetic acid. Gels were stored in the 5% acetic acid until a permanent photographic record could be made.

3.10 Western blotting

After electrophoresis, the gels were subjected to protein blotting onto nitrocellulose membranes to transfer the separated proteins. This was conducted using the basic methodology of Towbin et al (1979), and was carried out as follows. Briefly, the gels were blotted on to Hybond™-C super, supported nitrocellulose (Amersham International plc, Little Chalfont, Bucks, UK) by a semi-dry method using an LKB 2117 Multiphor II Electrophoresis System (Pharmacia LKB), used according to the manufacturers instructions, including the use of semi-dry transfer buffer (Tris, 48mM; glycine, 39mM; SDS, 0.0375%; methanol, 20%). Transfer was accomplished in 1 hour at a constant current of $0.8mA/cm^2$ of nitrocellulose membrane.

After blotting, the membranes were blocked to prevent non-specific binding of antibodies, with a 10% (w/v) milk powder solution (Marvel, Premier Brands, Adbaston, Staffs., UK) in Tris-buffered saline (TBS) (20mM Tris-HCl, pH 7.6; 150mM NaCl, from BDH). Blocking was carried out for 1 hour at room temperature, on a rocking table .

After blocking, the nitrocellulose membrane was incubated with the appropriate primary antibody solution, made up in 5% milk powder in TBS containing 0.1% Tween 20™ (Sigma). The membranes were again incubated on the rocking table, in this instance either overnight at 4°C, or for 3 hours at room temperature. After primary antibody incubation the membranes were rinsed twice with dH_2O and then washed 4 X with TBS containing 0.1% Tween-20 (TBST).

Next the blots were incubated with the appropriate secondary antibody, to allow protein detection following the ECL detection system (Amersham). The secondary antibodies used were species specific for the IgG of the species the primary antibody was raised in. The secondary antibodies were all conjugated to horseradish peroxidase and purchased from Amersham. The secondary antibody was diluted to the appropriate concentration with 5% milk powder in TBST, and the membranes were incubated in this solution for 1hour at room temperature, on a rocking table. The membranes were then washed as before, omitting the dH_2O rinsing step. The washed membranes were further processed using an enhanced chemiluminescent (ECL) detection technique from Amersham. This technique is a light-emitting, non-radioactive method for the detection of antigens, labelled directly, or indirectly with horseradish peroxidase (HRP) conjugated antibodies. This technique is based upon the ability of HRP to catalyze the oxidation of luminol, in the presence of hydrogen peroxide. Immediately following the oxidation, the luminol is in an excited state which decays to the ground state via a light emitting pathway. This light is then detected by photographic film.

Briefly, the ECL detection protocol involved the incubation of the membranes with the detection solutions provided in the ECL kit, for 1 minute and then exposing the membranes to photographic film (Hyperfilm-ECL, Amersham) for between 10 seconds and 30 minutes, depending on the intensity of the chemiluminescent signal. The films were then developed in X-ray development solution for approximately 1 minute, and fixed in X-ray fixative solution (both solutions were purchased in a concentrated form from Kodak Ltd., Liverpool, UK) for approximately 30 seconds. The films were then rinsed in dH_2O, dried, and photographed.

Chapter 4

Exogenous NADPH-induced reactive oxygen species (ROS) generation by human spermatozoa

4.1 Introduction

Although it has been demonstrated by many workers that human spermatozoa generate reactive oxygen species (ROS) (Alvarez *et al*, 1987; Aitken and Clarkson, 1987a and b; Iwasaki and Gagnon, 1992; Weese *et al*, 1993), the mechanism(s) by which they do so remain to be fully elucidated. It has been postulated that human spermatozoa generate reactive oxygen species via a mechanism that is similar in principle to the plasma membrane-bound NADPH oxidase complex of polymorphonuclear leucocytes. That human spermatozoa can generate the superoxide anion via a specialized mechanism, catalyzing the two electron reduction of molecular oxygen, employing NADPH as electron donor, is supported by some experimental evidence and, can also be inferred from the results of work carried out on the cellular mechanisms involved in the generation of reactive oxygen species by other, non-phagocytic, cell types (for review see Cross and Jones, 1991).

Let us deal firstly with the experimental evidence. Early work carried out by Aitken and Clarkson (1987a and b), showed that human spermatozoa generated the superoxide anion in response to the divalent cation ionophore, A23187. More recently, it has been shown that they also generate ROS in response to the phorbol ester, PMA (Aitken *et al*, 1992a and b). As both of these reagents are potent stimulators of the leucocyte NADPH oxidase, the authors postulated that this implied similarity between the ROS generating mechanisms of the two different cell types. The authors also presented data indicating that the source of electrons the spermatozoa employ are of a non-

mitochondrial origin, as disrupters of mitochondrial electron transport had no effect on A23187-induced superoxide anion generation (Aitken and Clarkson, 1987a and b). In the same study the authors also claimed that a burst of superoxide anion generation could be induced via the addition of exogenous NADPH to detergent-permeabilized spermatozoa, thus raising the possibility that NADPH is the electron donor employed *in vivo*,, in the intact cell. The fact that, when challenged with other possible substrates for superoxide generation, e.g. xanthine or various aromatic amino acids, the detergent-permeabilized spermatozoa did not generate superoxide, does go some way to demonstrating that the response to NADPH was a substrate specific one. Further confirmation that spermatozoa can employ NADPH as the electron donor for superoxide generation was supplied by Aitken and Ford (1988). In this abstract the authors presented data confirming the ability of detergent-permeabilized human spermatozoa to generate superoxide in response to exogenous NADPH, and also reported that mitochondrial inhibitors had no effect on this phenomenon. Further support for the hypothesis that NADPH is the electron donor *in vivo* was indicated by data showing a significant correlation between the activity of the hexose monophosphate shunt pathway (HMS) and both spontaneous and A23187-induced ROS generation. The HMS is the enzymatic pathway by which spermatozoa manufacture endogenous NADPH. Such a correlation implies a link between the production of reactive oxygen species and the availability of NADPH. Recently, this hypothesis has received further, much more solid support. Aitken and colleagues, examined the relationship between the levels of the HMS enzyme glucose-6-phosphate dehydrogenase (G6PDH), in human spermatozoa and their ability to generate ROS in response to the phorbol ester, PMA (Aitken *et al*, 1994b). This important paper showed that a significant relationship between the two parameters existed, i.e. that the magnitude of PMA-induced ROS generation by human spermatozoa was positively correlated with the levels of G6PDH

present in the spermatozoa. Since G6PDH occupies a pivotal position in controlling the rate of glucose flux through the HMS, its' activity can be used as a marker of overall HMS activity, and consequently, NADPH production. Thus these recent studies suggest that the capacity of human spermatozoa to generate ROS is directly dependent upon the cells' ability to generate NADPH.

Studies on numerous other non-phagocytic cells capable of ROS generation have all shown that an NADPH oxidase which is similar, and in some cases identical, to that of polymorphonuclear leucocytes, is responsible for the generation of the ROS. (Cross and Jones, 1991). This fact allows us to postulate that NADPH oxidase-like activity is employed by many cell types to generate ROS for specific biological purposes. It seems reasonable to assume that once a successful and controllable mechanism for ROS generation had been evolved, then this mechanism would be employed by all cell types which benefited from the ability to generate ROS. However, this is mere speculation and it remains for concrete evidence to be acquired to conclusively prove if this hypothesis is correct.

Work and progress in this area has been hindered by the fact that the spermatozoa of 'normal' donors generally generate very low levels of ROS, the postulate being that this is due to a paucity of endogenous substrate. If spermatozoa do generate ROS utilising an endogenous supply of NADPH supplied by the HMS, then one can appreciate that in a cell normally so devoid of cytoplasmic space, and hence cytoplasmic enzymes, the availability of NADPH is going to be a significant rate limiting factor controlling ROS generation. This may be a very important consideration when investigating the causes of pathological ROS generation by the spermatozoa of sub-fertile men. It has been shown that the level of peroxidative damage sustained by human spermatozoa is directly correlated with defects in the midpiece of the spermatozoa (Rao *et al,* 1989). It has also been postulated that this association exists because of the presence of excess residual cytoplasm in the defective

mid-piece (Aitken *et al*, 1994b). Greater amounts of residual cytoplasm would equate with higher levels of G6PDH in the cell, with a resultant increase in the generation of ROS, and the induction of lipid peroxidation. However, the majority of spermatozoa from 'normal' ejaculates do not exhibit mid-piece abnormalities associated with the retention of excess residual cytoplasm and hence do not produce ROS in high amounts. This low level of activity in normal specimens creates problems in terms of analysing the biochemistry and cell biology of ROS generation. With this problem in mind, it would be very useful for the study of ROS generation by spermatozoa, if it was possible to enhance the levels of ROS generation by normal, donor spermatozoa by side stepping the problem of limited substrate availability. In theory, this could be achieved by supplying the cells with an **exogenous** supply of substrate, i.e. NADPH. As has already been mentioned, this does seem to be possible in the case of detergent solubilized spermatozoa. However, it is not known if responses to NADPH could be obtained in the fully viable, intact spermatozoon. Again evidence from work carried out on ROS generation by other cell types would support this notion. Work on fibroblasts (Meier *et al*, 1989) and glomerular mesangial cells (Radeke *et al*, 1991; Satriano *et al*, 1993) has shown that supplying these cells with an exogenous source of NADPH, resulted in elevated levels of ROS generation. The work carried out in this chapter of the thesis reports the ability of intact spermatozoa to generate ROS utilising an exogenous supply of NADPH and attempts to elucidate the mechanism(s) involved, and to evaluate the *in vivo* significance of this phenomenon.

In the majority of experiments, ROS generation was monitored by chemiluminescence, a detection technique that is very sensitive, and capable of monitoring very low levels of ROS. A more conventional method of ROS detection, the superoxide dismutase inhibitable reduction of cytochrome c, was also employed, to quantify superoxide anion production accurately. Although

less sensitive than chemiluminescence for detecting ROS, this technique has the advantage that the ROS detected can actually be quantified, in terms of moles of superoxide anion generated.

Hopefully, the studies presented in this chapter will provide a useful experimental platform on which to base further work attempting to explain the cellular basis for ROS generation by human spermatozoa.

4.2 Materials and Methods

All experiments were performed at least three times and the results expressed as independent, representative, longitudinal analyses, i.e. individual traces of ROS generation, or as the means (±S.E.) of repeated measurements. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained, and the Fisher least significant difference (FLSD) computed at the 0.05 level of significance.

4.2.1 Cell preparation

Samples of pure populations of human spermatozoa were prepared as described in Chapter 3 of this thesis. The purity of the samples, i.e. freedom from contaminating leucocytes, was confirmed employing the FMLP provocation test; any samples responding positively to this test were either discarded and not used for any further analyses, or subjected to the Dyna-bead magnetic cell separation technique, until cell purity could be confirmed. All of the above techniques were carried out as described in the general methods chapter of this thesis. Pure suspensions of spermatozoa were incubated in BWW, at 37°C until use. Unless otherwise stated, all experiments were performed using the 100% Percoll® fraction of spermatozoa, adjusted to a concentration of $10x10^6$ cells/ml.

4.2.2 Exogenous nicotinamide adenosine dinucleotide diphosphate (NADPH) induced superoxide anion generation.

The ability of human spermatozoa to generate the superoxide anion when supplied with an exogenous electron donor, i.e. NADPH, was investigated along with the abilities of its' non-phosphorylated and non-reduced counterparts, i.e. NADH, $NADP^+$ and NAD^+. The ability of the alpha isomer of NADPH, usually non-active in reductive biosynthesis, was also investigated.

All pyridine nucleotides used were purchased from Sigma, and made up to the required concentration in PBS (Flow) immediately prior to use. Stock solutions of the nucleotides were made up at concentrations 10 fold higher than the required final working concentration, to allow for a 10 fold dilution on addition to the suspensions of spermatozoa. The usual protocol for lucigenin-dependent chemiluminescence was followed, to monitor superoxide anion generation. Once a steady signal was obtained, the luminometer was paused and the appropriate reagents added to the sperm suspensions at the required concentration. The luminometer was then re-started and the subsequent chemiluminescence monitored. Any further additions to the cell suspensions were carried out in a similar manner.

4.2.3 Quantification of superoxide anion generation

Superoxide anion generation was quantified spectrophotometrically, following the method of McCord and Fridovich (1969). This assay of superoxide anion production is based upon the ability of the superoxide anion to reduce cytochrome C and hence alter its absorbance at 550nM. The superoxide dismutase (SOD) inhibitable reduction of cytochrome C was measured on a Philips PU 8800 UV/VIS spectrophotometer (Pye Unicam Ltd, Cambridge, England) employing the turbid sample cuvette holders. In these experiments' spermatozoa were resuspended at a concentration of $10x10^7$/ml in BWW supplemented with PVA. 400µl of the spermatozoa suspension was placed in a

1ml disposable, plastic cuvette (BDH Chemicals) and 50µl of cytochrome C (Type VI from horse heart, Sigma) in PVA supplemented BWW, was added at a concentration of 1mM resulting in a final working cytochrome C concentration of 100µM when the cuvette contents were made up to 500µl with 50µl of BWW. The reference cuvette contained the same ingredients, except 50µl of SOD (bovine erythrocyte, 3910U/mg, E.C.1.15.1.1, Calbiochem) at a concentration of 2mg/ml in BWW, was substituted in place of the 50µl of BWW. The final working concentration of SOD was 196U/ml.

The absorbance, at 550nm, of the contents of the two cuvettes was monitored before and after the addition of 55µl of NADPH (20mM in PBS), to give a final working concentration of 2mM NADPH. The absorbance displayed by the spectrophotometer was that of the sample cuvette minus the absorbance of the reference cuvette. This gives a measurement of the SOD-inhibitable, and therefore superoxide anion-dependent, cytochrome C reduction. This data was then transformed to indicate the actual quantity of superoxide anion produced. This transformation of the data was achieved using the extinction coefficient for reduced cytochrome C, $\varepsilon = 21.1 mM^{-1}. cm^{-1}$. and an equation following the Beer-Lambert law:

$$A = \varepsilon b c$$

where A is the absorbance; ε is the extinction coefficient; b is the path length of the light passing through the sample cuvette, in this case 1cm.; and c is the quantity of superoxide anion (equivalent to the quantity of reduced cytochrome c, as 1 molecule of superoxide reduces 1 molecule of cytochrome c, see equation below).

$$\text{cyt c } (Fe^{3+}) + O_2^{\cdot -} \longrightarrow O_2 + \text{cyt c } (Fe^{2+})$$

The results were expressed as an individual spectrophotometer trace of a

representative experiment and also as the means ± S.E. of five separate experiments.

4.2.4 Investigations into the effect(s) of disrupters of mitochondrial electron transport on exogenous NADPH-induced superoxide anion generation.

The following inhibitors of mitochondrial electron transport were investigated for their ability to modulate exogenous NADPH-induced superoxide anion generation by human spermatozoa: antimycin A, rotenone, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and sodium azide (Sigma) and potassium cyanide (BDH). The above reagents were added individually to the sperm suspensions, after the addition of 500μM NADPH. Subsequent superoxide anion generation was monitored via lucigenin-dependent chemiluminescence, and any changes noted. Antimycin A, rotenone and CCCP were all made up in DMSO (BDH) to give stock solutions of 5mM, 1mM and 1mM respectively. Aliquots of these stocks were frozen until use. Potassium cyanide (KCN) was used as purchased, i.e. as a 5% solution. Sodium azide was made up in PBS to give a stock solution of 1mM. The appropriate volumes of the various reagents were then added to the spermatozoa suspensions, after the addition of 500μM NADPH, to result in the following final concentrations; 50μM antimycin A, 10μM rotenone, 10μM CCCP, 0.05%KCN, and 10μM sodium azide.

The results were expressed as the means ± S.E. of 3 separate experiments.

4.2.5 Investigations into the possible roles of other enzymes in NADPH-induced superoxide anion generation by human spermatozoa

The possibility that cellular components other than a novel 'NADPH oxidase' like enzyme system was involved in exogenous NADPH-induced superoxide generation by human spermatozoa was investigated using inhibitors of various enzymes capable of superoxide anion formation. The reagents, their

targets/actions, and the concentrations of each used are summarised in Table 4.0.

Table 4.0 Enzyme inhibitors investigated for effects of NADPH-induced superoxide anion generation by human spermatozoa.

Compound	Target	Concentration (s)
Sodium azide	Peroxidases Mitochondrial complex IV	10μM
Dicoumarol	DT-diaphorase	100μM
Sodium oxamate	Lactate dehydrogenase	1mM
Allopurinol	Xanthine oxidase	10mM

Briefly, the inhibitors were used as follows. Dicoumarol was purchased from Sigma, and a 10mM stock solution made with 0.1N sodium hydroxide solution. 4μl of this stock was added to the spermatozoa suspension prior to NADPH (500μM) addition. Subsequent lucigenin-dependent chemiluminescence was measured alongside that of control cell suspensions to which chemical vehicle, but no inhibitor, had been added. Similar protocols were followed for the other enzyme inhibitors used. Sodium azide, sodium oxamate and allopurinol were all purchased from Sigma, and made up in PBS to give stock solutions of the following concentrations; 1mM, 10mM, and 100mM respectively. The appropriate volumes were added to the cell suspensions, as for dicoumarol, to result in the following final concentrations; sodium azide 10μM; sodium oxamate 1mM; allopurinol 10mM. Lucigenin-dependent chemiluminescence in response to 500μM NADPH was measured and compared to that of control spermatozoa suspensions to which no

inhibitor had been added.

Results were expressed as the means ± S. E. of 3 separate experiments.

4.2.6 Impact of cell viability on NADPH-induced superoxide anion generation by human spermatozoa.

For the phenomenon of exogenous NADPH-induced superoxide anion generation to be of biological significance, it must be dependent on, and influenced by, the viability of the spermatozoa. To test this hypothesis the viability of the spermatozoa was altered under various experimental conditions, as follows:-

1) *Permeabilization by freeze-thawing*

In order to permeabilize the spermatozoa they were subjected to three cycles of freeze-thawing. This involved placing suspensions of spermatozoa, in plastic Falcon® tubes, in a rapid freezing bath composed of dry ice and methanol (BDH). The spermatozoa were incubated in the ice bath for a few minutes until the cell suspension was completely frozen. The spermatozoa were then thawed at room temperature, and subjected to two more cycles of freeze-thawing.

2) *Heat denaturation*

In order to denature endogenous sperm enzymes, suspensions of human spermatozoa were subjected to a heat inactivation process. This involved heating the spermatozoa to 57°C for thirty minutes. After heating and subsequent cooling the spermatozoa were resuspended in fresh PVA supplemented BWW, in order to avoid the consequences of any heat-induced changes in pH that may have occurred in the BWW.

3) *Membrane fixation*

Suspensions of spermatozoa were also treated with a protein fixative to

investigate whether fixation of the plasma membrane would affect electron transport from NADPH to the relevant sperm 'oxidase' component, and thence onto molecular oxygen. This was accomplished by the addition of 1µl of glutaraldehyde (BDH) to 400µl of the suspensions, resulting in a final working concentration of glutaraldehyde of 0.25% (v/v).

All the various spermatozoa suspensions were then investigated for their ability to generate the superoxide anion in response to 500µM NADPH, the responses being monitored by lucigenin dependent chemiluminescence as already described. Ten minutes before the end of the luminometer run, the suspensions had either 18U of SOD added or 1µl of glutaraldehyde (final concentration 0.25% v/v). Results were expressed as representative, individual luminometer traces and as the means ± S. E. of 3 separate experiments.

4.3 Results

4.3.1 Exogenous NADPH-induced superoxide anion generation.

Human spermatozoa readily generate the superoxide anion in response to the addition of exogenous NADPH to the culture medium (Figure 4.1). The superoxide generated could be monitored by lucigenin-dependent chemiluminescence and the kinetics of NADPH-induced superoxide generation consistently followed the same pattern, i.e. a sudden, sharp increase in the chemiluminescence signal upon addition of NADPH, which then continued to gradually increase in magnitude until reaching a plateau at which point the cells maintained a high, constant rate of superoxide production for several hours. The NADPH-induced chemiluminescent signal could be quenched upon the addition of 18 units of superoxide dismutase (SOD) to the suspension of spermatozoa, indicating that the chemiluminescence was indeed

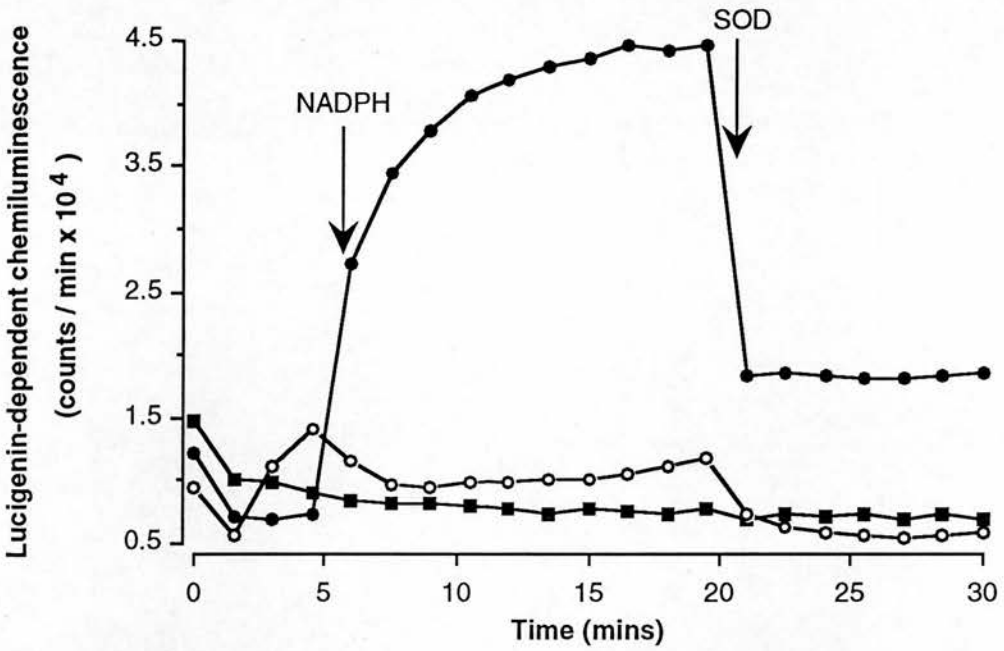

Figure 4.1 NADPH-induced superoxide anion generation by human spermatozoa, as monitored by lucigenin dependent-chemiluminescence. The identity of the ROS being monitored was confirmed by the ability of SOD (18U), to scavenge the chemiluminescent signal. Addition of NADPH to the spermatozoa resulted in a 10 fold increase in the chemiluminescent signal which could be almost totally scavenged with 18U SOD. The increase in chemiluminescence did not occur in controls, i.e. those with no spermatozoa present or without the addition of NADPH. Experiments were carried out in triplicate , and the traces are representative examples.
Spermatozoa + NADPH= —●— ; spermatozoa + PBS= —○— ; and BWW + NADPH= —■— .

due to the generation of superoxide (Figure 4.1).

Superoxide anion generation by human spermatozoa in response to exogenous NADPH was concentration dependent (Figure 4.2a and b), and overall, this NADPH concentration dependency was shown to be statistically significant (P = 0.001). A Km value for exogenous NADPH induced superoxide anion could not be obtained as no substrate saturation effect was observed (Figure 4.2b). Another feature of the response to NADPH was that the magnitude varied considerably from donor to donor, as illustrated in Figure 4.3a and b.

To confirm that a distinct, and substrate specific, enzymatic mechanism was being observed when spermatozoa were stimulated to generate superoxide with exogenous NADPH, the abilities of the two optical isomers of NADPH to stimulate such activity, were compared. Only the ß isomer, which is the form of NADPH that participates in enzymatic, reductive biosynthesis, significantly induced superoxide anion generation (Figure 4.4a); the α isomer was a very poor inducer of superoxide generation ($P<0.05$) (Figure 4.4b).

To determine the specificity of the response to exogenous NADPH further, the abilities of non-phosphorylated and non-reduced nicotinamide dinucleotides to induce superoxide anion generation by human spermatozoa were investigated. NADH induced superoxide anion generation by the spermatozoa at a level only approximately a third of that of the ß-NADPH, whilst $NADP^+$ and NAD^+ were almost completely incapable of inducing any superoxide generation (Figure 4.5a). The very low level of superoxide anion generation observed in response to these two non-reduced pyridine nucleotides was probably due to the $NADP^+$ and NAD^+ preparations being slightly contaminated with their reduced counterparts, or due to them being converted, intracellularly, to NADPH and NADH by endogenous sperm enzymes, e.g. enzymes of the glutathione cycle and lactate dehydrogenase respectively. Analysis of the group data revealed that the abilities of both the

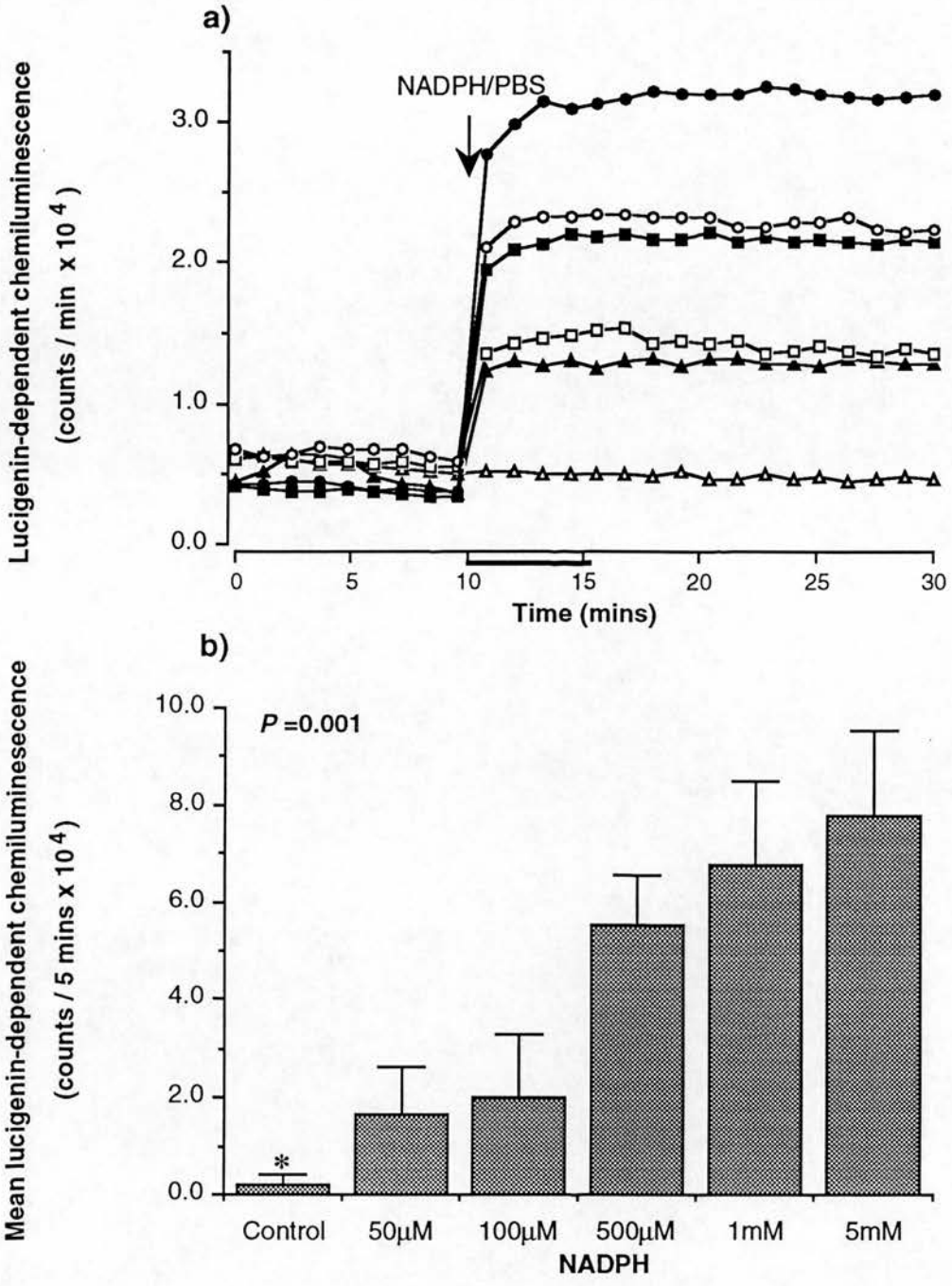

Figure 4.2 Concentration dependency of NADPH-induced lucigenin-dependent chemiluminescence by human spermatozoa. **a)** is an individual trace, representative of 8 separate dose response experiments, whilst **b)** depictes the means ±S.E. of the 8 separate experiments. Control chemiluminescence was significantly lower than that stimulated by all concentrations of NADPH, $*P < 0.05$. Overall, the concentration-dependent trend was statistically significant ($P = 0.001$).
Treatments: PBS control=—△— ;50µM NADPH= —▲— ; 100µM NADPH =—□— ; 500µM NADPH=—■— ; 1mM NADPH=—○— ; 5mM NADPH= —●— .

ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

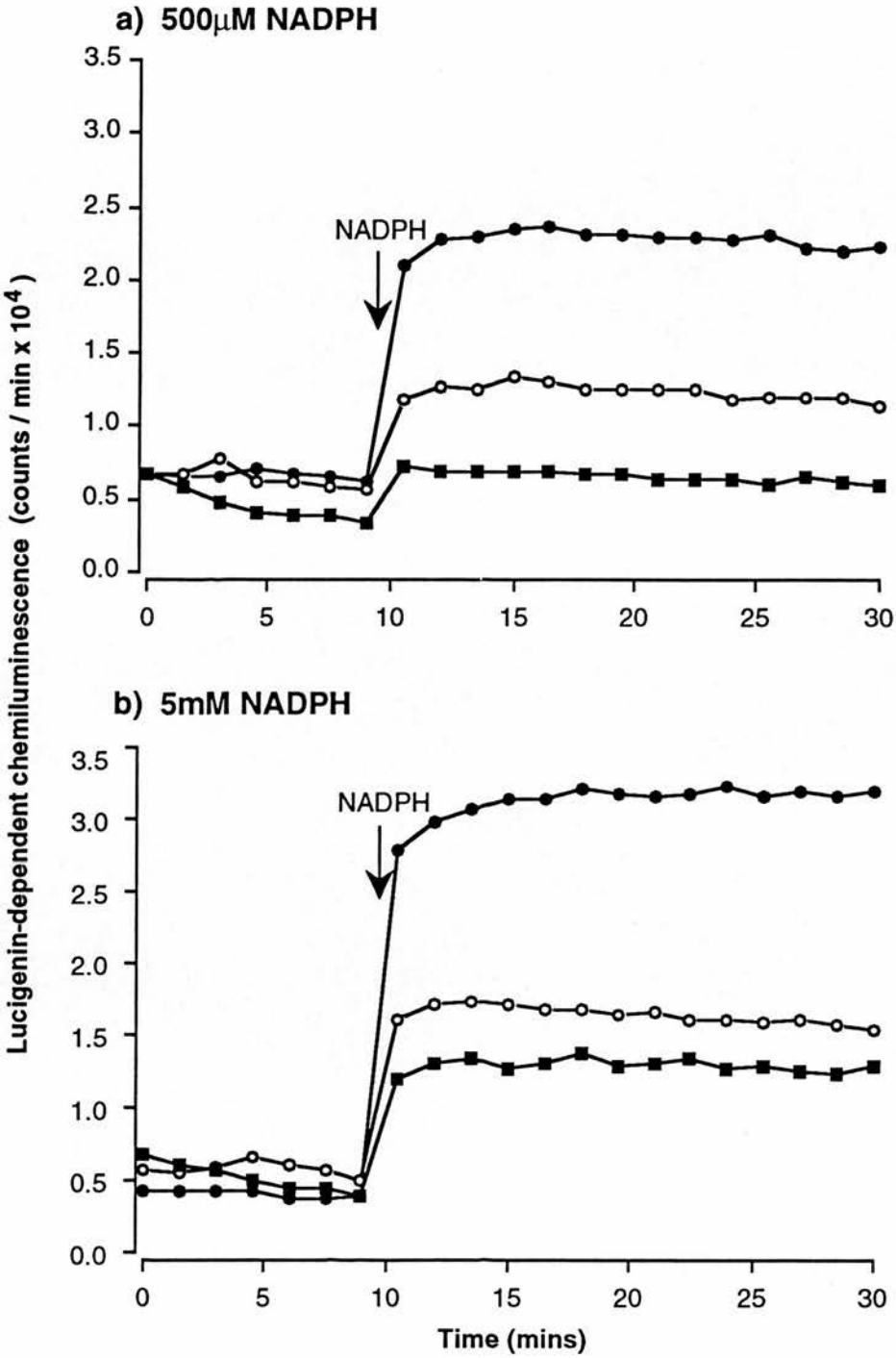

Figure 4.3 Demonstration of the donor to donor variation observed in the reponse to NADPH. Three individual donors were used to compare the lucigenin-dependent chemiluminescence response, to 500μM and 5mM NADPH, by human spermatozoa. Marked variations were observed between donors.
Donor 758=—●—; donor 655=—○—; and donor 333=—■—.

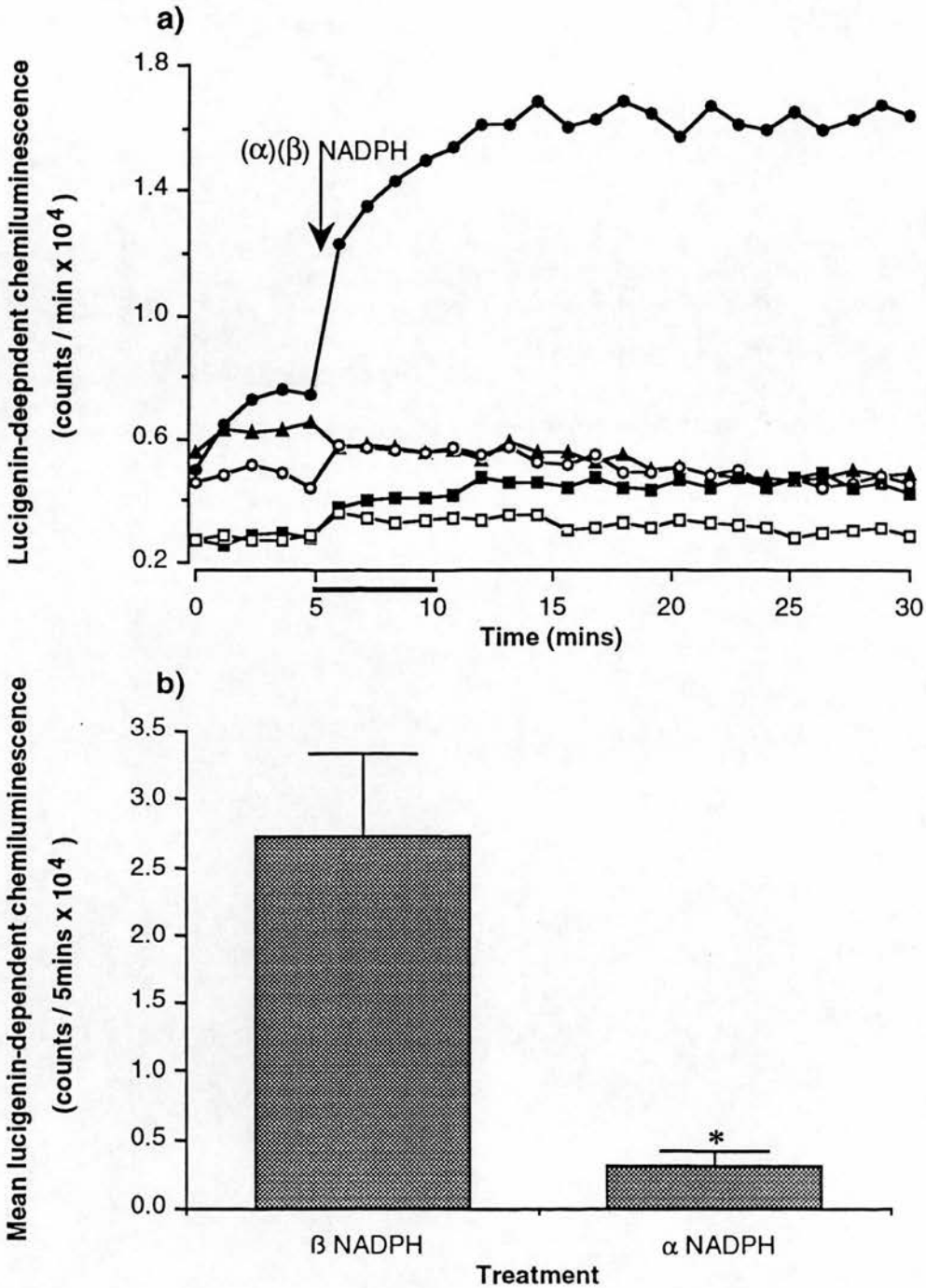

Figure 4.4. Comparison of the effects of different isomers of NADPH on lucigenin-dependent chemiluminescence by human spermatozoa. **a)** is an individual luminometer trace, whilst **b)** shows the means, ±S.E., of three separate experiments and demonstates that the reponse of human spermatozoa to α NADPH is significantly lower than the reponse to ß NADPH, $*P < 0.05$.

Sperm + ß NADPH = —●— ; sperm + α NADPH = —○— ;
sperm + PBS = —■— ; BWW + ß NADPH=—□— ; and BWW + α NADPH=—▲— .

ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

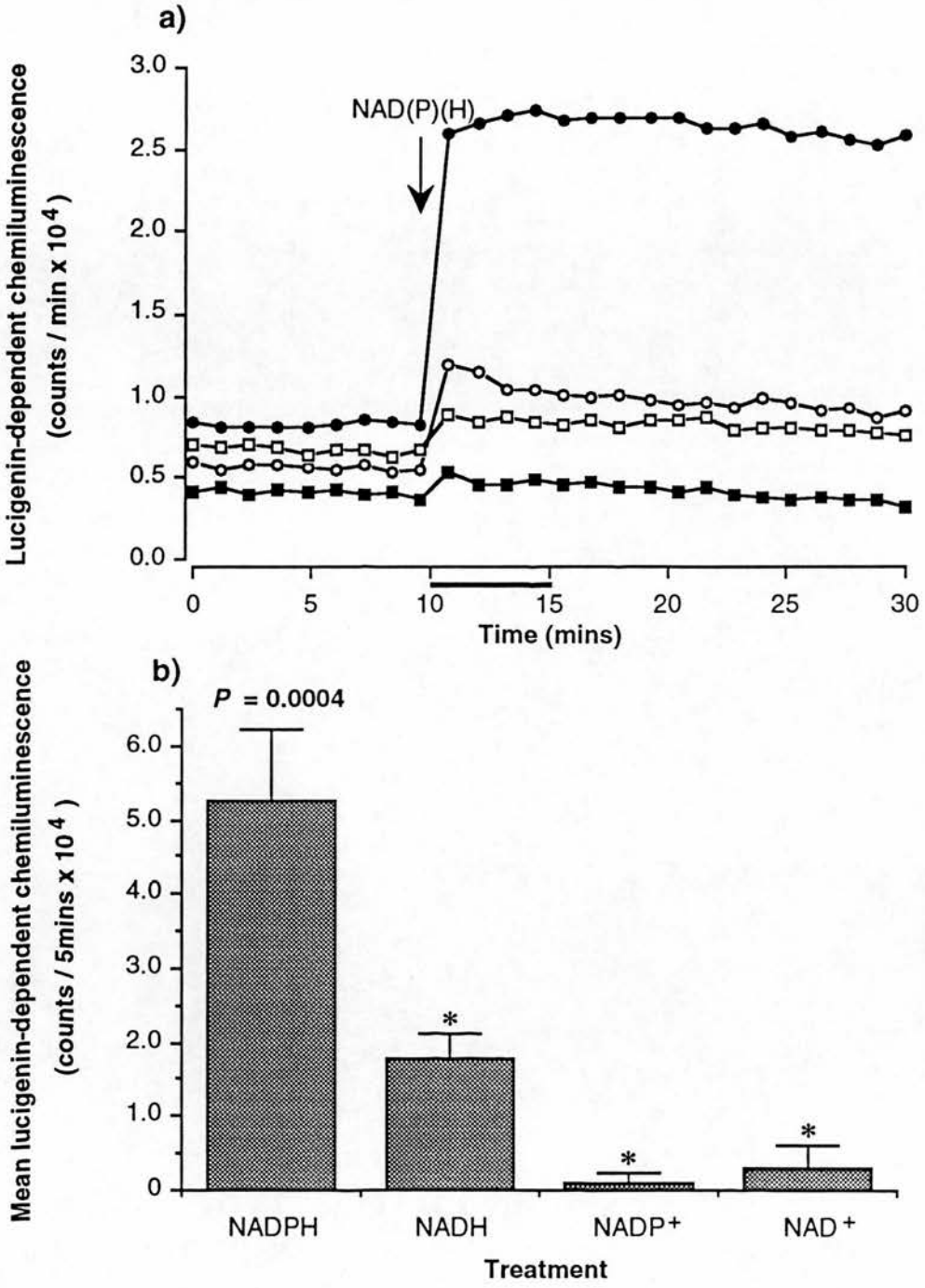

Figure 4.5 Comparison of the effects of various nicotinamide dinucleotides (500μM) on lucigenin-dependent chemiluminescence by human spermatozoa. **a)** is an individual luminometer trace, representative of 4 separate experiments, whilst **b)** shows the means ±S.E. of 4 separate experiments. The responses to NADH, $NADP^+$, and NAD^+ , were all significantly lower than the response to NADPH, $^*P < 0.05$. Overall, the reponses to the different adenine dinucleotides were significantly different from one another ($P = 0.0004$).
NADPH =—●— ; NADH =—○—; NADP =—■— ; and NAD =—□—.
ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

non-reduced pyridine nucleotides and NADH to induce superoxide anion generation by human spermatozoa, were significantly lower than the capacity of NADPH to stimulate this activity (Figure 4.5b, $P<0.05$).

4.3.2 Quantification of superoxide anion generation.

The actual quantity of superoxide generated by human spermatozoa in response to exogenously supplied NADPH was determined spectrophotometrically via the superoxide dismutase-inhibitable reduction of cytochrome c. The pattern of superoxide anion generation, as monitored by this method, seemed on superficial inspection, to closely reflect that observed when employing lucigenin-dependent chemiluminescence, i.e. an immediate and sharp increase in superoxide generation upon addition of the NADPH, followed by a constant and sustained level of superoxide generation (Figure 4.6). However, the kinetics of superoxide anion generation suggested by the two methodologies of detection, are, in reality, very different. From Table 4.1, it can be seen that although the initial rate of superoxide generation is very high, the final, constant and sustained rate is significantly much lower. This is in sharp contrast with the kinetics of superoxide generation observed when employing lucigenin-dependent chemiluminescence, in this instance the initial, high rate of superoxide generation is sustained throughout the course of the experiment.

The actual quantity of superoxide generated by an individual human sperm suspension, in response to the administration of 2mM NADPH is shown in Figure 4.6. This figure presents representative data from an individual experiment. The steady, and final rate of superoxide anion generation in this instance, was approximately 19 $nmols.min^{-1}.10^6$ $cells^{-1}$. Table 4.1 shows the means ± S.E. from 5 separate experiments of basal, maximal, and final rates of superoxide generation by human spermatozoa. Both the maximal and final rates of superoxide anion generation, in response to exogenous NADPH, are

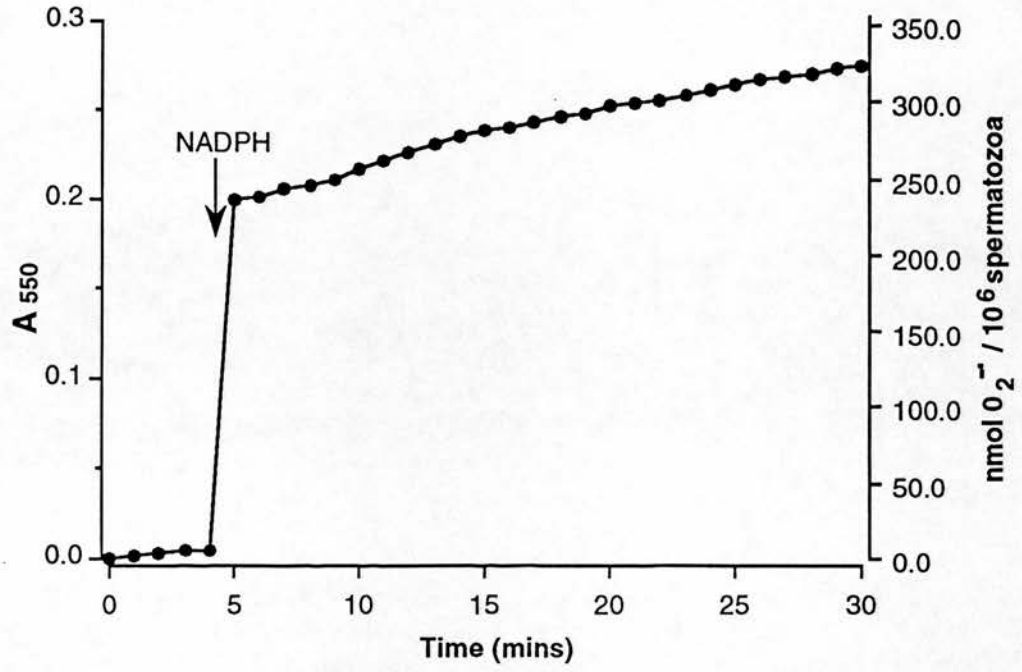

Figure 4.6 Quantification of NADPH-induced superoxide anion generation by human spermatozoa, as measured by the SOD-inhibitable reduction of cytochrome c. The change in absorbance, in response to NADPH (2mM), at 550nm, was followed and the generation of superoxide quantified using the extinction coefficient, 21.1 $mM^{-1}cm^{-1}$.

Table 4.1 Quantification of NADPH-induced superoxide anion generation by human spermatozoa, as monitored by the SOD-inhibitable reduction of cytochrome c.

Measurement recorded	Mean rate of superoxide formation (nmols / min / 10^6 spermatozoa ± S.E.)
Basal superoxide formation	3.01 (± 1.41)
Maximal superoxide formation	199.05 (± 18.25) *
Final superoxide formation	15.31 (± 1.94)*

The values quoted are the means ±S.E. of 5 separate experiments. The maximal and final rates of superoxide formation, in response to 2mM NADPH, were statistically higher higher than the control, spontaneous rate, *P <0.05. Overall, the rates were statistically different from one another (P = 0.001).

significantly higher than the basal rate, i.e. before NADPH administration ($P<0.05$ in both instances).

4.3.3 Effect of mitochondrial inhibitors.

Five disrupters of mitochondrial electron transport, acting at various points in the electron transport (Figure 4.7) pathway, were investigated for their abilities to influence exogenous NADPH-induced superoxide generation by human spermatozoa. Neither antimycin A, rotenone, CCCP, or sodium azide had any significant effect on NADPH-induced superoxide generation by human spermatozoa (Table 4.2). The effect of potassium cyanide could not be evaluated as this reagent interfered with the lucigenin-dependent chemiluminescence in an artefactual manner. Table 4.2 shows mean data ± S.E. from 3 separate experiments.

In all cases, differences in lucigenin-dependent chemiluminescence were not significant ($P>0.05$), indicating that the mitochondrial inhibitors had no significant affect on superoxide generation when compared with controls.

4.3.4 Roles of non-NADPH oxidase enzymes

A variety of enzyme inhibitors active against potential sources of ROS, including azide (peroxidases), dicoumarol (diaphorase), oxamate (lactate dehydrogenase) and allopurinol (xanthine oxidase) were examined for their effects on NADPH-induced superoxide generation. None of the compounds tested exerted any significant effect on exogenous NADPH-induced superoxide generation by human spermatozoa. The results from these experiments are shown in table form, depicting the means ±S.E. of 3 separate experiments (Table 4.3).

In all instances, no significant differences were observed between control levels of superoxide generation by the spermatozoa and the levels observed when the spermatozoa were incubated with the various inhibitors ($P > 0.05$ for

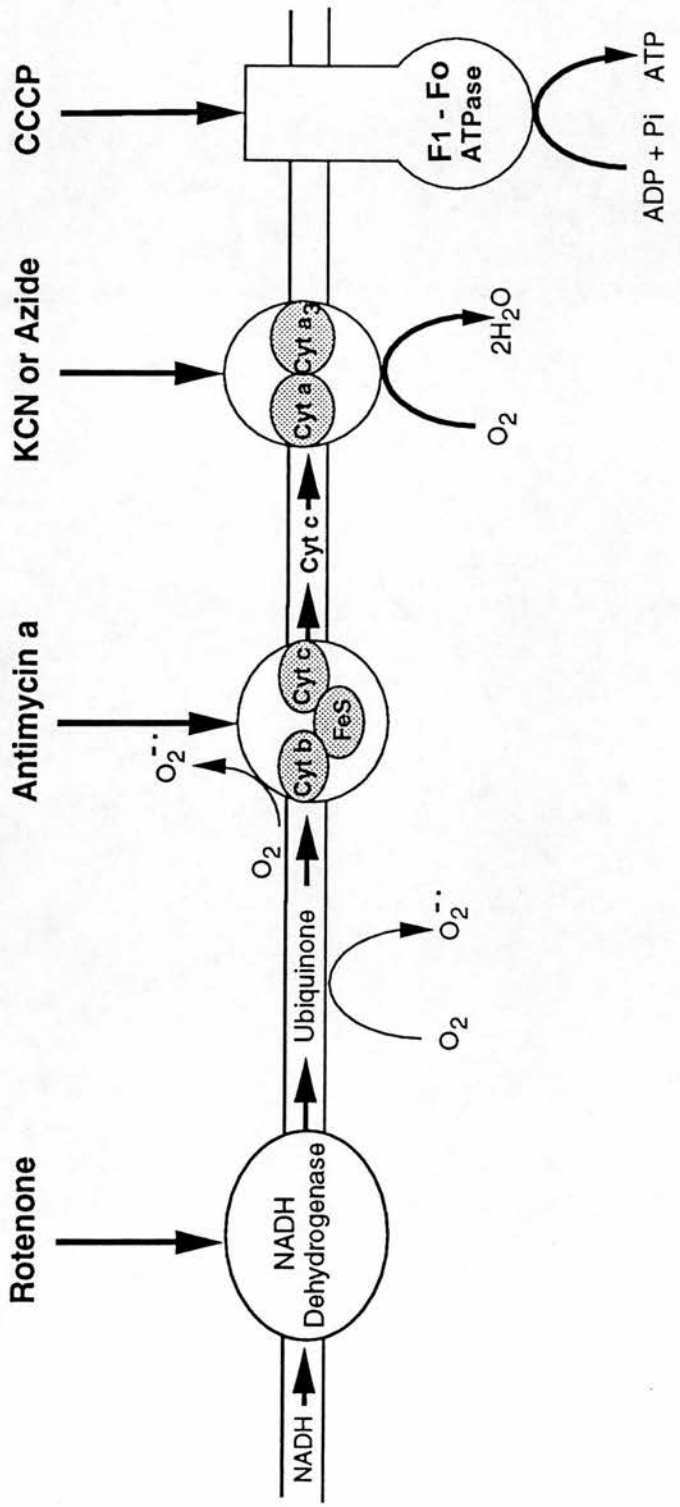

Figure 4.7 Diagramatic representation of the mitochondrial electron transport chain. In this model the bold arrows indicate the direction of the flow of electrons, whilst the fainter arrows show the potential sites of superoxide production, due to leakage of electrons. The large, vertical arrows indicate the sites of action of the various mitochondrial inhibitors mentioned in the text.

Table 4.2 Lack of an effect of inhibitors of mitochondrial electron transport on exogenous NADPH-induced superoxide anion generation by human spermatozoa

Mitochondrial inhibitor tested	Treatment	Lucigenin -dependent chemiluminescence (mean integrated counts x 10^4 over 5 minutes ± S.E.)
Antimycin A	Control	10.173 (± 2.162)
	50μM antimycin A	10.535 (± 2.494)
Rotenone	Control	9.624 (± 1.677)
	10μM rotenone	8.967 (± 2.258)
CCCP	Control	14.989 (± 8.768)
	10μM CCCP	12.609 (± 6.970)
Sodium azide	Control	8.616 (± 0.983)
	10μM sodium azide	7.690 (± 1.538)

The values quoted are the means ±S.E. of three separate experiments. The integration period was the 5 minutes immediately after addition of NADPH (500μM). The control values are the NADPH responses from cells which were not treated with any inhibitor. None of the inhibitors tested had any significant effect on NADPH-induced chemiluminescence by human spermatozoa.

Table 4.3 Lack of an effect of inhibitors of various potential enzymatic sources of ROS on exogenous NADPH-induced superoxide anion generation by human spermatozoa

Inhibitor tested	Treatment	Lucigenin -dependent chemiluminescence (mean integrated counts x 10^4 over 5 minutes ± S.E.)
Sodium oxamate	Control	4.882 (± 2.042)
	1mM Sodium oxamate	5.204 (± 2.092)
Dicoumarol	Control	4.300 (± 1.707)
	100μM Dicoumarol	4.128 (± 1.115)
Sodium azide	Control	8.616 (± 0.983)
	10μM Sodium azide	7.690 (± 1.538)
Allopurinol	Control	7.536 (± 2.057)
	10mM Allopurinol	7.189 (± 1.656)

The values quoted are the means ±S.E. of three separate experiments. The integration period was the 5 minutes immediately after addition of NADPH (500μM). The control values are the NADPH responses from cells which were not treated with any of the enzyme inhibitors. None of the inhibitors tested had any significant effect on NADPH-induced chemiluminescence by human spermatozoa.

all the inhibitors tested).

4.3.5 Effect of spermatozoa status.

The impact of cell viability on exogenous NADPH induced superoxide generation by human spermatozoa was also investigated. The results obtained from these experiments are shown in Figures 4.8a and b. Samples of spermatozoa which were subjected to three cycles of freeze-thawing, i.e. membrane permeabilization, showed a substantial increase in their response to the administration of exogenous NADPH, compared to intact, non-freeze-thawed spermatozoa. This increase was approximately 9-fold and was significantly greater than the response of the non-freeze-thawed spermatozoa ($P<0.05$). The response of the frozen-thawed cells was susceptible to superoxide dismutase inhibition, indicating that the increased lucigenin-dependent chemiluminescence observed was indeed due to an increase in the detection of superoxide anion.

Sperm suspensions that were subjected to 57°C for thirty minutes did not generate any superoxide anion in response to exogenous NADPH, nor did those fixed with 0.25% glutaraldehyde prior to NADPH addition. The inhibition in both instances was statistically significant, $P<0.05$. Glutaraldehyde also inhibited superoxide anion generation by the cells which had been subjected to the freeze-thawing process of membrane permeabilization.

4.4 Discussion

The realization that spermatozoa generate reactive species is not a recent one. Over fifty years ago the Scottish andrologist John McLeod demonstrated that the viability of human spermatozoa could be supported by the addition of catalase to the medium, implying that in their steady-state, human

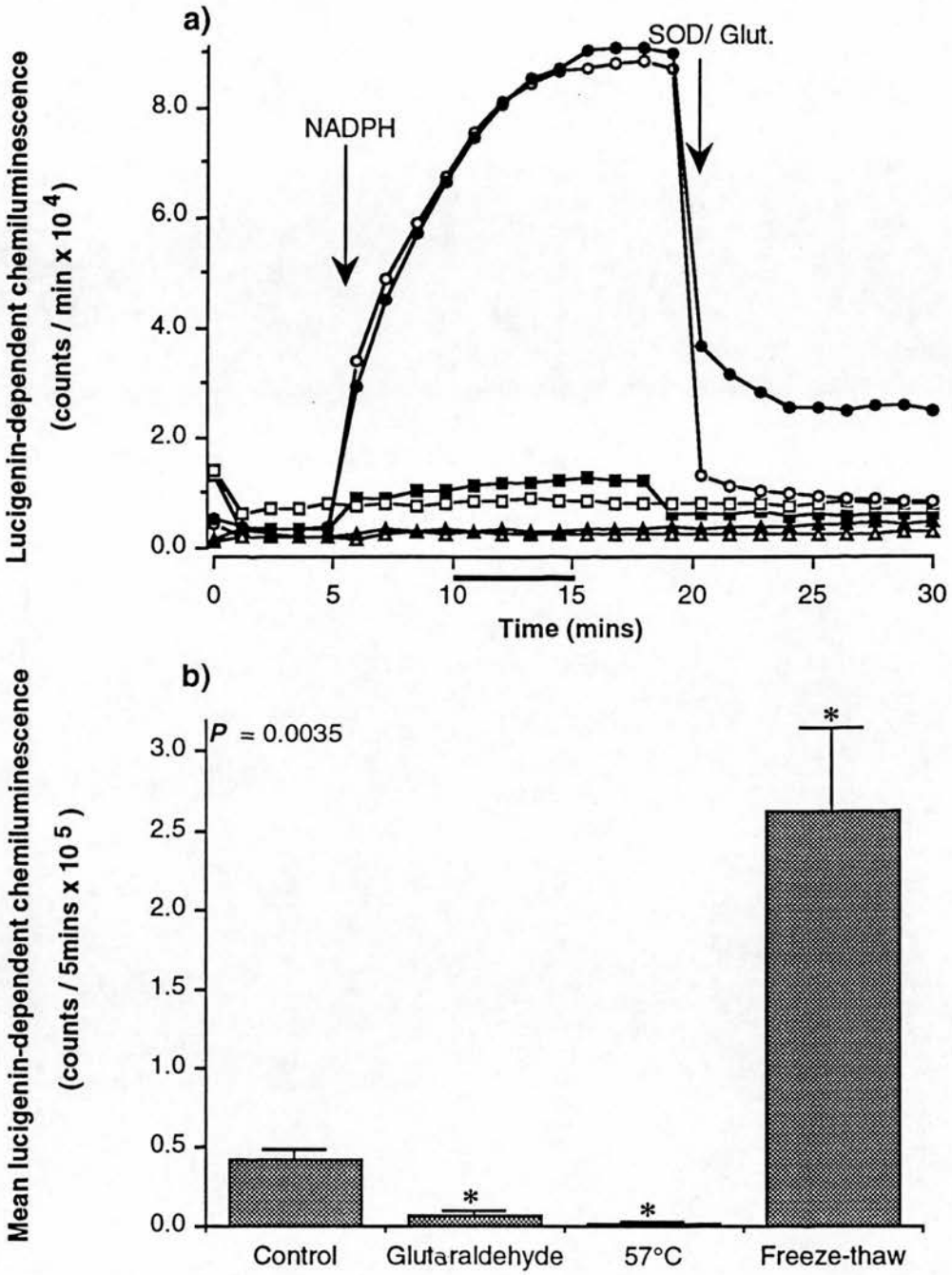

Figure 4.8 Effect of various cell-status, altering experimental procedures on NADPH-induced (500μM), lucigenin-dependent chemiluminescence. SOD (18U) or glutaraldehyde (0.25%) was added towards the end of the experiment, in some instances. **a)** is a representative trace, of 3 separate experiments, whilst **b)** shows the means ± S.E. of the 3 separate experiments. All the treatments had a significant effect, in comparison to the control, $*P < 0.05$. Overall, the treatments gave significantly different results (P =0.0035).
Treatments: control= —■—; Glut (0.25%)= —□— ; F-T + Glut = —○—; F-T + SOD = —●— ; 57°C= —▲— ; 57°C= —△— .

ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

spermatozoa generate hydrogen peroxide which is deleterious to them (McLeod, 1943). A few years after the publication of this work, Tosic and Walton published data demonstrating that bovine spermatozoa were indeed capable of generating ROS, specifically hydrogen peroxide (Tosic and Walton, 1946). In more recent years, both rabbit spermatozoa (Holland *et al*, 1982; Alvarez and Storey, 1982) and mouse spermatozoa (Alvarez and Storey, 1984) have been shown to generate ROS, including the superoxide anion and hydrogen peroxide. Human spermatozoa have also been shown to generate ROS, primarily the superoxide anion, which then secondarily dismutates to hydrogen peroxide under the influence of intracellular superoxide dismutase (Aitken and Clarkson 1987a and b; Alvarez *et al*, 1987).

However, although much work has now been carried out investigating ROS generation by spermatozoa, it still remains for the biochemical mechanisms by which they do so to be fully elucidated, this being particularly true in the case of the human spermatozoon. The cellular mechanisms involved in the generation of ROS by bovine spermatozoa have been postulated to involve the deamination of aromatic amino acids (Tosic and Walton, 1950), and ROS generation by rabbit spermatozoa has been shown to be the result of the leakage of electrons from the mitochondrial electron transport chain (Holland *et al*, 1982). Neither of these two cellular phenomena appears to be involved in ROS generation by human spermatozoa (Aitken and Clarkson, 1987a and b).

Work carried out to date has suggested that human spermatozoa generate ROS via a mechanism which has similarities, in principle, to the NADPH oxidase of phagocytic leucocytes, utilizing NADPH as the electron donor to facilitate the one electron reduction of molecular oxygen to the superoxide anion. Early work carried out by Aitken and Ford (1988) showed that human spermatozoa did generate superoxide upon administration of an exogenous supply of NADPH. However, this work was carried out employing detergent-

solubilized spermatozoa and it has, until now, remained for concrete evidence to be presented showing that functionally viable, intact spermatozoon can generate ROS utilising NADPH as the electron donor. The results presented in this chapter clearly indicate that this is indeed the case, but the results also raise some fundamental issues which must be addressed.

The very fact that human spermatozoa generate superoxide in response to an exogenous supply of NADPH requires some careful consideration. *In vivo* endogenous NADPH is generated by the hexose monophosphate shunt (HMS) (Aitken and Ford, 1988), a cytoplasmic enzyme pathway. It follows that the NADPH produced by the HMS must be cytoplasmic in its' location also. Thus, *in vivo*, ROS generation relies on intracellular NADPH, suggesting or implying that the enzyme(s) responsible for the ROS generation must have an intracellular NADPH binding site. The intracellular nature of the NADPH binding site could manifest itself by being present on the cytosolic side of the sperm plasma or acrosomal membranes, or by the ROS generating enzyme or enzyme complex being, itself, cytosolic. Since NADPH is thought to be largely membrane impermeant, it remains to be determined how this compound could interact with a putative intracellular NADPH binding site to induce superoxide anion generation. However, these results do not set a precedent, as other cell types have been shown to behave in a similar fashion, i.e. generate the superoxide anion in response to an exogenous supply of NADPH (Meier *et al*, 1991; Radeke *et al*, 1991). There are at least two plausible explanations as to how exogenous NADPH might induce superoxide generation, these explanations not necessarily being mutually exclusive.

The first possible explanation is based on the premise that the NADPH is somehow entering the cell. Differences in the permeability of the plasma membrane of the spermatozoa to NADPH may exist due to such factors as varying lipid composition and prior peroxidative damage. The lipid composition of the sperm plasma membrane changes during such

physiological events as epididymal maturation and capacitation (Zaneveld *et al*, 1991; Drobnis, 1993) and this may result in changes in the plasma membranes' permeability to NADPH. Peroxidative damage or modification to the sperm plasma membrane can also render it more permeable to various molecules, including NADPH (Jones and Mann, 1973; Mann and Lutwak-Mann, 1973; Alvarez and Storey, 1982). The hypothesis that NADPH is somehow entering the cell is also supported by the fact that permeabilization, induced by repeated cycles of freeze-thawing, resulted in a dramatic increase in the ability of the spermatozoa to generate superoxide in response to exogenous NADPH, relative to non-permeabilized cells. Of course, the observed increase may be due to some effect of the freeze-thawing process other than cell permeabilization, one possibility being of a loss of intracellular superoxide dismutase which would otherwise scavenge the superoxide. Loss of intracellular superoxide dismutase has been shown to occur during cryopreservation of human spermatozoa (Lasso, *et al*, 1994). It is possible that the NADPH enters the cells in a non-passive way, e.g. a specific or even non-specific active transport mechanism being employed; however there is no evidence to support such a mechanism in spermatozoa or any other cell type. The fact that high doses of NADPH are required to induce superoxide generation is also indicative of a passive process being involved. The second, and less plausible, of the explanations for exogenous NADPH induced superoxide generation by human spermatozoa is that the oxidase responsible for ROS generation has a NADPH binding site which has an extracellular domain as well as an intracellular one.

Whichever of the above described possibilities is correct, it is important to point out that the phenomenon of exogenous NADPH-induced ROS generation is not one solely exhibited by the human spermatozoon. In fact numerous reports indicate that other cell types, shown to possess NADPH oxidase 'like' activity, generate ROS in response to the administration of exogenous

exogenous NADPH. These exogenous NADPH-responsive cells include glomerular mesangial cells (Satriano *et al,* 1993; Radeke *et al,* 1991) and fibroblasts (Meier *et al,* 1989) both of which have been reported to respond to exogenous NADPH with a sustained burst of superoxide anion generation. All the authors concerned speculate that the NADPH, unable to traverse the intact plasma membrane of the cells, gains entry via areas of the plasma membrane which have incurred sub-lethal peroxidative damage, rendering them 'leaky'. The authors also suggest the possibility that the NADPH oxidase of these cells has an NADPH binding site on the external side of the plasma membrane. Significantly, the inactivated, unassembled NADPH oxidase of phagocytic leucocytes does not generate ROS in response to exogenous NADPH, although the active assembled oxidase does, and the NADPH binding site of the NADPH oxidase is known to be located on the internal side of the plasma membrane of these cells (Smith et al, 1989).

Do these results, and the comparisons with other cell types, imply that the oxidase of human spermatozoa, unlike the NADPH oxidase of phagocytes, requires no prior activation, but is simply regulated by substrate, i.e. NADPH, availability? It has already been pointed out that the ability of human spermatozoa to generate ROS in response to the phorbol ester, PMA, appears to be dependent upon HMS activity (Aitken *et al,* 1994b), suggesting that substrate availability is a very important rate limiting factor in ROS generation by human spermatozoa. Another possibility is that the exogenous NADPH somehow activates the system responsible for ROS generation, as this has been postulated for the NADPH oxidase of phagocytic leucocytes (Fujii and Kakinuma, 1991). These authors suggest that NADPH not only acts as a specific reductant in superoxide generation by the NADPH oxidase, but also as a cofactor for the conformational modification of the NADPH oxidase system to a superoxide generating state. This could be the situation in human spermatozoa. It is possible that in situations of low substrate availability, as

exist in the 'normal spermatozoon', the amount of NADPH present is not sufficient to effect this conformational modification, but that PMA can somehow alleviate this requirement. When the spermatozoa are supplied with exogenous NADPH, a conformational change may occur, possibly altering the affinity of the binding site for NADPH, and stimulates increased levels of superoxide anion generation.

Looking at the kinetics of superoxide generation by human spermatozoa in response to exogenous NADPH, the observed pattern, i.e. of a continual and sustained release of superoxide anion, seems to reflect that of fibroblasts (Meier *et al*, 1989), endothelial (Sundqvist, 1991)) and mesangial cells (Radeke *et al*, 1991) as opposed to the short burst of ROS generation, lasting only a few minutes, observed upon stimulation of the NADPH oxidase of phagocytic leucocytes. Similarly, the actual quantity of superoxide generated by human spermatozoa is more in line with the low yields of superoxide anion generation associated with non-phagocytic cells, than with the comparatively high amounts produced by activated phagocytes. However, the kinetics of the SOD-inhibitable cytochrome c reduction (Figure 4.6) reflect those observed when active, cell-free leucocyte NADPH oxidase is stimulated to generate ROS with NADPH (Cross *et al*, 1984). A Km value for NADPH could not be determined as there was no substrate saturation effect observed, and it is interesting to note, that in the literature, no Km values for NADPH are reported for the other cell types in which the phenomenon of exogenous NADPH induced superoxide generation has been shown. However, lack of observable substrate saturation of an enzyme is a very rare occurrence, one of the few known enzymes to exhibit this behaviour being catalase (Chanci *et al*, 1979); the rate of the enzyme-substrate complex association and dissociation being so rapid as to render enzyme active site availability not rate limiting. It would be interesting to know if this was the case for the superoxide forming oxidase of human spermatozoa, though it is not the situation in the NADPH oxidase of

phagocytic leucocytes, the reported Km value for NADPH of this enzyme complex varies, but it is in the region of 30-40μM (Rotrosen *et al*, 1992). In the human spermatozoon, it could be the case that in the intact cell, sufficient NADPH cannot gain access to the oxidase NADPH binding site in order to stimulate the maximum possible superoxide formation, i.e. that in the intact cell substrate availability will always be the rate limiting factor and the enzyme involved will never become substrate saturated due to the limited permeability of the spermatozoon to NADPH. A way around this problem would be to develop a 'cell free' system with which to study the true kinetics of the superoxide generating system of spermatozoa, thus removing the constraints that the limited permeability of the plasma membrane imposes. However, the problem could not be overcome by simply permeabilizing the cells as this would also allow the NADPH access to the mitochondria and possibly bring into play other cellular components, outwith an NADPH oxidase-like system, capable of ROS generation.

To explore further the hypothesis that human spermatozoa are generating the superoxide anion, in response to NADPH via a specific enzymatic mechanism, experiments were performed to investigate the stereo-isomer specificity of NADPH-induced response. The results obtained from comparing the abilities of the α and ß isomers of NADPH to induce superoxide anion formation supported the notion that stereo-specificity does exist in this instance. The α isomer of NADPH is not active as a coenzyme (Palmer, 1985) and therefore it should be unable to act as an electron donor for enzymatic superoxide anion generation. This was shown to be the case. Only the ß NADPH was capable of stimulating superoxide generation, implying an enzymatic basis for the NADPH-dependent reduction of oxygen to superoxide and ruling out any possible involvement of non-specific electron donor actions of NADPH.

Preference for the phosphorylated nicotinamide adenine dinucleotide

over its non-phosphorylated counterpart, NADH, was also shown, in keeping with the substrate specificity of the NADPH oxidase of phagocytic leucocytes and other cells. Like the phagocyte NADPH oxidase, ROS generation by human spermatozoa could be supported by NADH, but the level of superoxide anion generated was only a fraction of that produced when the spermatozoa were incubated with the same concentration of NADPH. This appears to be the case for all the 'NADPH oxidase' like systems described in other cells, i.e.. that NADPH is the preferred electron donor. This situation is in keeping with the hypothesis that in reactions of reductive biosynthesis, e.g. superoxide formation, NADPH is the required coenzyme, serving as a donor of hydrogen ions or hydride ions, whilst in reactions of oxidative degradation and energy production, NADH is the coenzyme necessary to provide electrons (Kaufman, 1993). In fact it is quite strange that the two coenzymes are even slightly interchangeable, as it has been recognised for quite some time that enzymes are generally very specific for one coenzyme or the other (Hopkins, 1976). However, the cellular location of the NADPH -oxidase or NADPH oxidase like enzymes, may negate the need for such an absolute substrate specificity, as NADH is mainly mitochondrial in location and therefore unlikely to come in contact with a plasma membrane-bound enzyme, such as the NADPH oxidase. Of course, it cannot be ruled out that the spermatozoon possesses greater permeability to NADPH than NADH, but this issue will only be resolved after the development of a pure, cell free preparation of NADPH oxidase-like activity from human spermatozoa.

In terms of the mitochondrion, spermatozoa possess these organelles and so have a relatively high potential for oxidative phosphorylation, which can result in electron leakage and radical formation (Cummins *et al*, 1994). As has already been stated, it has been shown that generation of ROS by rabbit spermatozoa involves the leakage of electrons from the mitochondrial electron transport chain (Killian *et al*, 1985). This has also been shown to be the case in

other cell types, e.g. the macrophage (Rembish and Trush, 1994), and so deserves further consideration as a potential source of ROS in human spermatozoa.

The mitochondria of all cells are possible sources of ROS, due to the very nature of the electron transfer reactions that take place in these organelles. No chemical reaction can be 100% efficient and inevitably some electrons 'leak' out of the electron transport chain and may effect the partial reduction of molecular oxygen, forming the superoxide anion, or other radical species, which can then participate in redox-cycling reactions. Mitochondria are not only potential sources of the superoxide anion but may also generate hydrogen peroxide, through the action of superoxide dismutase (Boveris and Chance, 1973) which exists in this organelle as the manganese form of the enzyme. It is now realised that mitochondrial ROS generation is a very important cellular phenomenon, and that it is probably one of the key components of the ageing process, the age related loss of cellular integrity being a direct result of the peroxidative damage to proteins and DNA which ROS can initiate (Harman, 1981; Stadtman, 1992; Linnane *et al*, 1989; Short, 1993). For all of these reasons, it is important to determine whether or not ROS generation by human spermatozoa has any mitochondrial component.

Early work on ROS generation by human spermatozoa has shown that mitochondrial electron leakage does not significantly contribute to the ROS generated by these cells, including spontaneous ROS generation and that observed in response to the divalent cation ionophore, A23187 (Aitken and Clarkson 1987a and b). An absence of mitochondrial involvement in the NADPH-induced superoxide anion generation by detergent permeabilized human spermatozoa has also been reported (Aitken and Ford, 1988) but this study is not comparable to that conducted here as in this instance, intact, fully viable cells were employed. Thus experiments were designed to determine the role, if any, of mitochondrial electron leakage in the exogenous NADPH-

induced superoxide anion generation by intact, human spermatozoa.

The possible leakage of electrons from the mitochondrial electron transport chain might lead to the direct reduction of molecular oxygen to the superoxide anion or to the creation of other radical species (e.g. $NADP^{\cdot}$) that might then yield superoxide on reaction with molecular oxygen (see equation 1).

$$NADP^{\cdot} + O_2 \longrightarrow NADP^{+} + O_2^{-\cdot} \quad (1)$$

The need to evaluate the mitochondrial contribution to the NADPH-induced response is also emphasised by the recent demonstration that lucigenin can enter the mitochondria of macrophages and detect superoxide generated therein (Rembish and Trush, 1994). Thus any superoxide anions generated by sperm mitochondria might have contributed to the overall lucigenin-dependent chemiluminescence observed when investigating NADPH-induced ROS generation by human spermatozoa.

To investigate the above possibilities, various inhibitors of mitochondrial electron transport were employed, and their abilities to modulate superoxide anion generation by human spermatozoa in response to exogenous NADPH, assessed. Five such compounds, acting at various points in the mitochondrial electron transport chain (see Figure 4.7), were tested and four of these had no significant effect on ROS generation, as monitored by lucigenin-dependent chemiluminescence. Rotenone, a complex I inhibitor which blocks electron flow between ubiquinone and NADH-dehydrogenase, was used to investigate the possible role of electron leakage from these sites of the mitochondrial electron transport chain. Two conflicting opinions exist as to what the result of rotenone inhibition should be, if electron leakage from complex I is playing a role in ROS production. Rembish and Trush (1994) report that a reduction in superoxide anion generation would be observed whilst Turrens and McCord

(1990) suggest that an increase in superoxide anion generation would result. In this instance this discrepancy is of no real importance, as when human spermatozoa were incubated with rotenone no significant change in superoxide anion generation was observed. Similar results were obtained with antimycin A, a complex III inhibitor that blocks the respiratory electron transport chain between cytochrome b and ubiquinone. Thus, electrons originating from ubiquinone do not contribute to NADPH-induced superoxide anion generation by human spermatozoa, although ubiquinone was shown to be the site of electron leakage in rabbit sperm mitochondria, accounting for the generation of ROS by the spermatozoa of this species (Chapman *et al*, 1985).

CCCP, another uncoupler of oxidative phosphorylation, specifically inhibiting ATP synthase, also had no effect on NADPH-induced superoxide anion generation by human spermatozoa. Finally, inhibitors of mitochondrial complex IV were investigated, these being potassium cyanide and sodium azide, both blocking the flow of electrons between cytochrome c and cytochrome oxidase. These two inhibitors should, if electrons from complex IV are involved in superoxide anion generation, result in a reduction in superoxide anion generation (Rembish and Trush, 1994). Unfortunately the effect of potassium cyanide on superoxide generation could not be evaluated as addition of this compound to the chemiluminescence probe lucigenin resulted in a spurious, non-biological increase in spontaneous luminescence. Moreover, this was the case, even in control experiments where spermatozoa were absent. This effect of potassium cyanide has been reported previously (Rembish and Trush, 1994), though the chemistry behind such an effect is unknown. No such artefactual chemiluminescent signals were observed when sodium azide was added to lucigenin, and so the lack of any effect of this mitochondrial inhibitor on spermatozoa ROS generation can be taken to indicate that electrons from complex IV have no role in this phenomenon. In conclusion, the data generated by these studies strongly suggest that

superoxide anion generation by human spermatozoa, in response to the administration of exogenous NADPH, is not in any way the consequence of electron leakage from the mitochondrial electron transport chain.

It is known that some enzymes which are not primarily catalysts of ROS generation do, under some circumstances, generate ROS. In order to establish whether the activities of such enzymes were contributing to exogenous NADPH-induced superoxide anion generation by human spermatozoa a series of experiments were conducted involving inhibitors of such enzymes, i.e. peroxidase, diaphorase, lactate dehydrogenase and xanthine oxidase.

Peroxidases are enzymes that catalyze the conversion of hydrogen peroxide to water and oxygen, with the concomitant oxidation of another compound. Peroxidases are also known to catalyze the aerobic oxidation of pyridine nucleotides, i.e. NADH and NADPH (De Sandro *et al,* 1991). The reactions of peroxidase-dependent NADPH oxidation are dependent on the presence of a mediating compound and this can take the form of the hydrogen peroxide adduct compound I (equation 2), the latter being one of the products of the peroxidase-dependent degradation of hydrogen peroxide. The reaction product of NADPH oxidation is $NADP^{\bullet}$ (equation 3). Another molecule of $NADP^{\bullet}$ is then formed by the reaction of NADPH with compound II (equation 4) and the superoxide anion is generated when $NADP^{\bullet}$ reacts with molecular oxygen (equation 5).

$$\text{Peroxidase} + H_2O_2 \longrightarrow \text{Compound I} \qquad (2)$$

$$\text{Compound I} + \text{NAD(P)H} \longrightarrow \text{Compound II} + \text{NAD(P)}^{\bullet} + H^{+} \qquad (3)$$

$$\text{Compound II} + \text{NAD(P)H} \longrightarrow \text{peroxidase} + \text{NAD(P)}^{\bullet} + H^{+} \qquad (4)$$

$$2\text{NAD(P)}^{\bullet} + 2O_2 \longrightarrow 2\text{NAD(P)}^{+} + 2O_2^{-\bullet} \qquad (5)$$

Peroxidases are present in mammalian spermatozoa, including glutathione peroxidase in human spermatozoa (Alvarez and Storey, 1989; Aitken *et al*, 1992b), which serves as a protective enzyme involved in the scavenging of hydrogen peroxide. In addition, there is a report of numerous different peroxidases in bovine spermatozoa (Pavlova *et al*, 1994). Thus, it is possible that peroxidase(s) present in human spermatozoa are responsible for, or contributing to, the observed superoxide anion generation by these cells, in response to NADPH. To investigate whether or not this was the case, sodium azide, an inhibitor of sperm peroxidase (Aitken *et al*, 1992b), was added to cells induced to generate superoxide via the addition of exogenous NADPH. Addition of sodium azide had no significant effect on lucigenin-dependent chemiluminescence, suggesting that peroxidases are not involved in the mechanism by which exogenous NADPH induces superoxide anion generation by human spermatozoa.

Another enzyme present in human spermatozoa capable of generating superoxide is NAD(P)H-oxioreductase or diaphorase (Gavella and Lipovac, 1992). This enzyme is a flavoprotein, and in the liver protects against the cytotoxicity and mutagenicity of quinone derived free radicals, as well as those derived from vitamin K dependent protein carboxylation; both processes involving the two electron oxidation of either NADH or NADPH (Lind *et al*, 1990). Diaphorase enzyme activity can also lead to superoxide anion generation (Fisher and Gutierrez, 1991) and diaphorase has also recently been shown to possess nitric oxide synthase activity (Hope *et al*, 1991). The presence of this enzyme in human spermatozoa was first shown by Caldwell *et al*, (1976) and it was shown to be distinct from the diaphorases of other cells and tissues and to exhibit genetic polymorphism. More recently, Gavella and Lipovac (1992) have shown this enzyme in human spermatozoa to be present in the midpiece of the spermatozoon and to be integrated with the mitochondrial respiratory system. It has also been shown that the levels of diaphorase

activity in sub-fertile men correlate well with their ability to generate ROS (Gavella and Lipovac, 1993). This association could be due to an overall increase in the level of cytoplasmic enzymes, including those of the HMS responsible for the generation of endogenous NADPH in spermatozoa. To determine whether the sperm diaphorase was responsible for superoxide generation in response to exogenous NADPH, the inhibitor dicoumarol, an antagonist of vitamin K (Lind *et al*, 1990), was added to the spermatozoa. Dicoumarol had no effect on NADPH induced superoxide anion generation and so it is probable that diaphorase plays no role in exogenous, NADPH-induced superoxide anion generation by human spermatozoa. However, it cannot be ruled out that the sperm diaphorase is not susceptible to dicoumarol inhibition.

Another sperm enzyme which has the potential for ROS generation is lactate dehydrogenase (LDH) (Bielski and Chan, 1973). This enzyme catalyzes the reversible inter-conversion of lactate to pyruvate, employing NADH as the coenzyme electron donor. However, in the presence of trace amounts of the superoxide anion, a $NAD^{\cdot}$ radical may be formed (equation 6) which can then go on to reduce molecular oxygen to the superoxide anion (equation 7). This reaction is a self-perpetuating, radical generating, chain reaction. The literature states that only NADH is capable of participating in such a reaction and that NADPH is completely inactive in this instance (Bielski and Chan, 1973). However, the possibility that LDH does play a role in NADPH-induced superoxide generation deserves investigation in the light of reports indicating that there exists a strong correlation between elevated levels of LDH in human spermatozoa and infertility (Noguera Velasco *et al*, 1993; Orlando *et al*, 1994). It has also been reported that a positive correlation exists between the level of LDH C_4 and the ability of human spermatozoa to generate the superoxide anion (Gavella and Lipovac, 1993).

$$LDH + NADH \longrightarrow LDH\text{-}NADH \quad (6)$$

$$LDH\text{-}NADH + O_2^{-\bullet} + H^+ \longrightarrow LDH\text{-}NAD^{\bullet} + H_2O_2 \quad (7)$$

$$LDH\text{-}NAD^{\bullet} + O_2 \longrightarrow LDH\text{-}NAD^+ + O_2^{-\bullet} \quad (8)$$

Sodium oxamate is a competitive inhibitor of LDH (Novoa *et al*, 1959) and this compound has been shown to inhibit the oxidation of NADH in the LDH system, and hence inhibit superoxide anion formation (Chan and Bielski, 1980). The addition of sodium oxamate to human spermatozoa had no effect on exogenous NADPH induced superoxide anion generation by human spermatozoa, thus ruling out the participation of LDH in this cellular phenomenon.

One further cellular enzyme known to generate the superoxide anion is xanthine oxidase. This enzyme, present in many tissues, was first shown to generate the superoxide anion in 1968 by McCord and Fridovich. This enzyme uses xanthine as substrate and reacts with water and oxygen forming urate, superoxide and hydrogen ions. The xanthine/xanthine oxidase system is a very powerful superoxide generator and is frequently used as a source of free radicals in *in vitro* experiments (de Lamirande and Gagnon, 1993a and b; Aitken *et al*, 1992c; Wu *et al*, 1992). A powerful inhibitor of xanthine oxidase is allopurinol (Halliwell and Gutteridge, 1986). In this thesis allopurinol was used to investigate whether or not xanthine oxidase was involved in exogenous NADPH-induced superoxide anion generation by human spermatozoa. Addition of this compound to spermatozoa, stimulated to generate superoxide with NADPH, did not effect the response, ruling out the possibility that xanthine oxidase is involved in NADPH-induced superoxide anion generation by human spermatozoa.

In summary, it appears that human spermatozoa generate superoxide

anion in response to exogenous NADPH via a mechanism, probably enzymatic, that is distinct from many of the already characterized enzymes, present in spermatozoa and other cell types, that are known to be capable of generating ROS. Some similarities do appear to exist between the ROS generating system in spermatozoa and the NADPH oxidase of leucocytes, though it remains to be seen if the two systems are actually identical to one another.

To address further whether an enzymatic mechanism is involved in the transfer of electrons from NADPH to molecular oxygen, experiments were carried out investigating the effect of cell viability and membrane integrity on this phenomenon. If an enzyme or enzyme complex is involved in the generation of superoxide generation by human spermatozoa, then it is rational to assume that if the spermatozoa are subjected to a process which will denature their endogenous enzymes then this should result in the abolition of superoxide generation. This was shown to be the case. When human spermatozoa were incubated at 57°C for 30 minutes, in order to heat denature all enzymes, the spermatozoa did not generate the superoxide anion in response to the administration of exogenous NADPH. Since most enzymes are susceptible to heat inactivation, this result points to the possible involvement of an enzymatic mechanism in superoxide anion generation by human spermatozoa. The loss of cell viability does not in itself lead to a loss of NADPH-induced superoxide anion generation, since spermatozoa which were killed via repeated cycles of freeze-thawing still responded to NADPH administration. In fact, when subjected to repeated freeze-thawing cycles, the spermatozoa's response to NADPH increased almost 10-fold. This could have a number of possible causes. Firstly, the increase could be a result of the freeze-thawing process permeabilizing the cells and thus allowing a greater amount of NADPH to enter the cells and effect superoxide anion generation, as suggested earlier. Another possible factor could be the loss, or inactivation, of

sperm components responsible for down-regulating superoxide anion generation, e.g. superoxide dismutase (Lasso *et al.*). It is also possible that increased superoxide anion generation was due to changes in the ionic composition of the internal milieu of the spermatozoa. In particular, it is conceivable that changes in the levels of cytosolic calcium might have caused an increase in superoxide anion generation, the rational behind this suggestion stemming from the fact that the activity of the NADPH oxidase of phagocytic leucocytes is known to be partly calcium dependent (James *et al,* 1981; Hallett *et al,* 1990).

Treatment of human spermatozoa with glutaraldehyde resulted in complete inhibition of NADPH-induced superoxide anion generation. For electron transfer to occur between NADPH, the spermatozoa oxidase and molecular oxygen, the enzymes involved, need to be in an active form. Glutaraldehyde induces cross-linking between amino groups and causes denaturation and inactivation of proteins, including enzymes, and thus prevents electron transfer. The glutaraldehyde results would thus support those obtained in the heat inactivation studies, indicating that the ability of human spermatozoa to generate the superoxide anion in response to exogenous NADPH, is due to an enzymatic process. Work carried out investigating the effect of glutaraldehyde on superoxide generation by guinea pig polymorphonuclear leucocytes has produced a rather contrasting set of results (Katayama *et al* , 1990; Sakane *et al,* 1987). In these experiments it was shown that glutaraldehyde only inhibited superoxide generation in cells in which the NADPH oxidase had not been previously activated. In cells which had been activated prior to glutaraldehyde treatment, the ability to generate superoxide was maintained, in fact prolonged. The authors postulate that the glutaraldehyde is acting via a membrane stabilisation mechanism, i.e. in the inactivated cells glutaraldehyde-dependent stabilization of the plasma membrane prevents the assembly of the various oxidase components, whilst in

the activated cells, the stabilization process prevents disassembly of the components, thus maintaining the activity of the NADPH oxidase complex. It seems in this instance that the glutaraldehyde is not actually preventing the electron transfer reactions from occurring and is not denaturing the proteins/enzymes involved (i.e. the components of the NADPH oxidase). It should be noted however, that the concentration of glutaraldehyde used in this instance was very low, 0.05% compared with the 0.25% used in the experiments described in this thesis. This concentration, of glutaraldehyde, 0.05%, would be very unlikely to denature proteins and possibly be unlikely to even cause any protein cross-linking. The authors appreciate this and state that they are unsure how the glutaraldehyde is exerting its effects. However, they do point out that the glutaraldehyde has no effect on the Km values for NADPH of the oxidase and speculate that this reflects that enzymatic activity has not been affected. It would, perhaps, be interesting to look at the effect of 0.05% glutaraldehyde on NADPH-induced superoxide generation by human spermatozoa, though it is probable that no effect would be seen due to the apparent, constantly assembled/active nature of the spermatozoa oxidase.

4.5 Summary and conclusions

In conclusion, the results presented in this thesis support the hypothesis that human spermatozoa generate the superoxide anion, utilizing NADPH as electron donor, via a specialized cellular mechanism that appears to be specifically intended for ROS generation. The mechanism appears to be enzymatic in nature, and shows some similarities to the NADPH oxidases of phagocytic leucocytes, fibroblasts and glomerular mesangial cells. However, it remains for further work to be carried out on the biochemical characteristics of the NADPH oxidase of human spermatozoa, in order to fully understand its activity and the cellular mechanisms involved in its' function. Work

attempting to clarify these matters will be carried out in the forthcoming chapters of this thesis.

Putting the biochemical mechanisms for superoxide generation aside for one moment, the discovery that intact human spermatozoa can be artificially stimulated to generate ROS through the addition of exogenous NADPH, will be of great value in elucidating the influences of ROS generation on the cell biology of viable human spermatozoa.

Chapter 5

Analyses of the cellular mechanisms and components involved in reactive oxygen species generation by human spermatozoa.

5.1 Introduction

Previous investigations have shown that human spermatozoa generate reactive oxygen species (ROS) (Aitken and Clarkson, 1987a and b; Alvarez *et al*, 1987). This phenomenon appears to possess some biochemical features of the NADPH oxidase of phagocytic leucocytes. The NADPH oxidase-like activity of human spermatozoa is dependent on a supply of electrons from NADPH, and uncouplers of oxidative phosphorylation have no effect on ROS generation (previous chapter and Aitken and Clarkson, 1987b), thus excluding the possibility of a mitochondrial origin of ROS in human spermatozoa. Inhibitors of xanthine oxidase, diaphorase and lactate dehydrogenase, all enzymes that have been shown to be capable of generating ROS, have no effect on ROS generation by human spermatozoa, indicating the existence of a possibly novel and unique, specialized enzymatic system in human spermatozoa responsible for ROS production.

In order to explore the possibility that human spermatozoa generate ROS by an NADPH oxidase-like system, it would be valuable to elucidate the cellular mechanisms involved in the control of ROS generation by human spermatozoa, and identify the cellular components involved.

5.1.1 Control of ROS generation

One pathway by which the NADPH oxidase of phagocytic leucocytes becomes activated is known to involve protein kinase C (PKC) (Tauber, 1987-review). The involvement of this protein kinase has been demonstrated by the ability of the PKC activating phorbol ester, PMA (phorbol 12-myristate 13-acetate) to

stimulate ROS generation by these cells. It has also been demonstrated that specific inhibitors of PKC block ROS generation by leucocytes (Koenderman *et al*, 1989; Tamaoki *et al*, 1986; Fujita *et al*, 1986; Curnutte *et al*, 1994, Kramer *et al*,1989), providing further support for the involvement of PKC in NADPH oxidase activation. The role of PKC in ROS generation appears to be to phosphorylate a cytosolic component of the NADPH oxidase, which subsequently leads to the assembly and activation of the oxidase, finally resulting in superoxide anion generation. It has already been demonstrated that the phorbol ester, PMA stimulates human spermatozoa to generate ROS (Aitken *et al*, 1992a and b; Aitken and Buckingham, 1992; Krausz *et al*, 1992; Aitken *et al*, 1993a; Krausz, *et al*,1994), the PMA exerting its' effect specifically, i.e. due to its' ability to activate PKC, since non-PKC activating phorbol esters such as 4α-phorbol 12,13-didecanoate, do not stimulate ROS generation by human spermatozoa. Thus, the stimulation of ROS generation by human spermatozoa may involve the phosphorylation, by PKC, of one or more of the cellular components involved in this activity. Using specific inhibitors of PKC, and serine/threonine specific phosphatase inhibitors, studies were undertaken to determine whether or not protein phosphorylation is involved in the regulation of ROS generation by human spermatozoa.

To date, no components of the ROS generating system of human spermatozoa have been identified. This is not the situation in the case of the leucocyte NADPH oxidase, of which numerous components have now been identified, isolated and characterized. Of great significance has been the identification of the flavoprotein and cytochrome components of the oxidase (now known to jointly form a flavocytochrome), components that are involved in the transfer of electrons from NADPH to molecular oxygen. As human spermatozoa also employ NADPH as electron donor for their ROS generation, it would be very interesting, and of great importance, to determine whether the ROS generating system of human spermatozoa also possesses cytochrome,

flavoprotein and/or flavocytochrome components. Work carried out on the leucocyte NADPH oxidase has lead to the identification of specific inhibitors of the flavoprotein component of the NADPH oxidase (Cross and Jones 1986; Cross, 1990). Addition of these inhibitory compounds to the oxidase results in the abolition of ROS generation, presumably through prevention of electron transfer from the NADPH to the oxidase, and thence to molecular oxygen. Experiments described in this chapter will use such inhibitors in an attempt to determine whether there is a flavoprotein involved in ROS generation by human spermatozoa.

5.1.2 Identification of cellular components involved in ROS generation by human spermatozoa

Although human spermatozoa generate ROS using NADPH as the electron donor, it remains to be determined whether the NADPH oxidase-like system of human spermatozoa and its' phagocytic counterpart are structurally as well as functionally similar. The structural relationships between the phagocyte NADPH oxidase and the NADPH oxidases of some other, non-phagocytic cells have already been evaluated, and this has revealed that the components of the NADPH oxidase are not exclusively expressed by phagocytic leucocytes and that in fact B-lymphocytes (Hancock *et al*, 1991), fibroblasts (Meier *et al*, 1991) and glomerular mesangial cells (Radeke *et al*, 1991) also express certain components.

Many, although possibly not all, of the cellular components of the phagocyte NADPH oxidase have been identified and data pertaining to these published. The first component to be identified was the unusually low potential, cytochrome b of the oxidase (Segal and Jones, 1978), i.e. cytochrome b_{558}, also known as cytochrome b_{559} and cytochrome b_{-245}. The cytochrome is the terminal component of the electron transfer chain of the oxidase, and is composed of two components; an α and a β subunit (Parkos *et al*, 1987). The α

subunit is the smaller of the two, being approximately 22kD in size, whilst the β subunit is much larger at around 91kD. Depending on the extent of the ß sub-units glycosylation, it has reported molecular weights of between 69-100kD (Parkos *et al*, 1987; Segal, 1987). After the discovery of cytochrome b_{558}, it was quite a long time before it was finally resolved as to which, if any, of the subunits possessed the NADPH binding site. However, a consensus is now beginning to emerge, with researchers in the field agreeing that the β subunit of the cytochrome b_{558} possesses the NADPH binding site, and that it is, in fact, a flavocytochrome, containing the FAD as well as the NADPH binding site (Segal *et al*, 1992; Rotrosen *et al*, 1992; Sumimoto *et al*, 1992; Doussiere *et al*, 1993). Both the subunits of the cytochrome have been isolated, antibodies raised against them, and their amino acid and nucleotide sequences determined (Royer-Pokora *et al*, 1986; Parkos *et al*, 1988). Two other NADPH oxidase components that are well characterised are the cytosolic factors, $p47^{phox}$ and $p67^{phox}$. Both of these cytosolic components are translocated to the plasma membrane upon stimulation of the phagocyte and this results in oxidase assembly and activation (Clark *et al*, 1990). The smaller of the two, $p47^{phox}$, is a phosphoprotein and is phosphorylated on its' serine and threonine residues by PKC, before associating with the cytochrome b_{558} (Segal *et al*, 1985). $p67^{phox}$ also associates with the cytochrome b_{558} upon oxidase activation and close analysis of the amino acid sequence of this protein has shown sequence motifs with strong homology to those found in non-catalytic *src*-related tyrosine kinases (Leto *et al*, 1990). Both of these cytosolic components have been fully sequenced and antibodies raised against them (Volpp *et al*, 1989; Lomax *et al*, 1989; Leto *et al*, 1990). The fifth oxidase component which is also well characterized is the small GTP binding protein, $p21^{rac\ 1}$ (Knaus *et al*, 1992; Abo *et al*, 1991). This protein is again involved in the assembly and activation of the oxidase and associates with the cytochrome

during the formation of the active NADPH oxidase complex (Quinn *et al*, 1989; Abo *et al*, 1991; Knaus *et al*, 1991).

The unusual characteristics of the cytochrome, the development of antibodies against the known oxidase components and knowledge of the amino acid and nucleotide sequences of the components, all enable investigations to be carried out into whether or not these components, or very similar ones, are present in human spermatozoa. Such studies should allow us to determine whether the functional and the structural properties of the ROS generating system in human spermatozoa are indeed similar to those of the NADPH oxidase, first described in phagocytic leucocytes.

In order to determine the structural relationship, if any, between the phagocyte NADPH oxidase and its' spermatozoon counterpart, various biochemical and molecular strategies have been employed. Thus, spectral analyses have been conducted on isolated, human sperm plasma membrane preparations, in order to determine whether the low potential b-type cytochrome is present in these cells (Cross *et al*, 1981). This technique has been used with much success in determining the presence of cytochromes in many cells and membranes, and due to the unusual spectral features of the cytochrome b_{558}, the technique is especially useful for determining its' presence in a mixture of cytochromes. The cytochrome b_{558} has a midpoint potential markedly lower than any other b-type cytochrome in mammalian cells and shows an easily recognisable, characteristic spectrum (Figure 5.1) (Cross *et al*, 1981). In its' reduced form the cytochrome b_{558} shows peaks at 528nm and 558nm, with a Soret peak at 428nm that shifts to 418nm when the protein is oxidised. The cytochrome b_{558}, like other low potential cytochromes forms a complex with carbon monoxide (CO), and this feature can be exploited, to analyse further, the difference spectra exhibited by this cytochrome. Upon binding of CO, the cytochrome b_{558} spectrum exhibits a strong absorption band at 418nm, which discriminates it from similar

cytochromes. Thus, by performing low temperature difference spectrophotometry upon human sperm plasma membranes it should be possible to ascertain whether or not the plasma membrane of these cells contains the low potential cytochrome b_{558} associated with the NADPH oxidase of phagocytic leucocytes. Similar analyses have also been conducted on preparations of general sperm membranes, in order to investigate the possibility of a non-plasma membrane location of the cytochrome b_{558}.

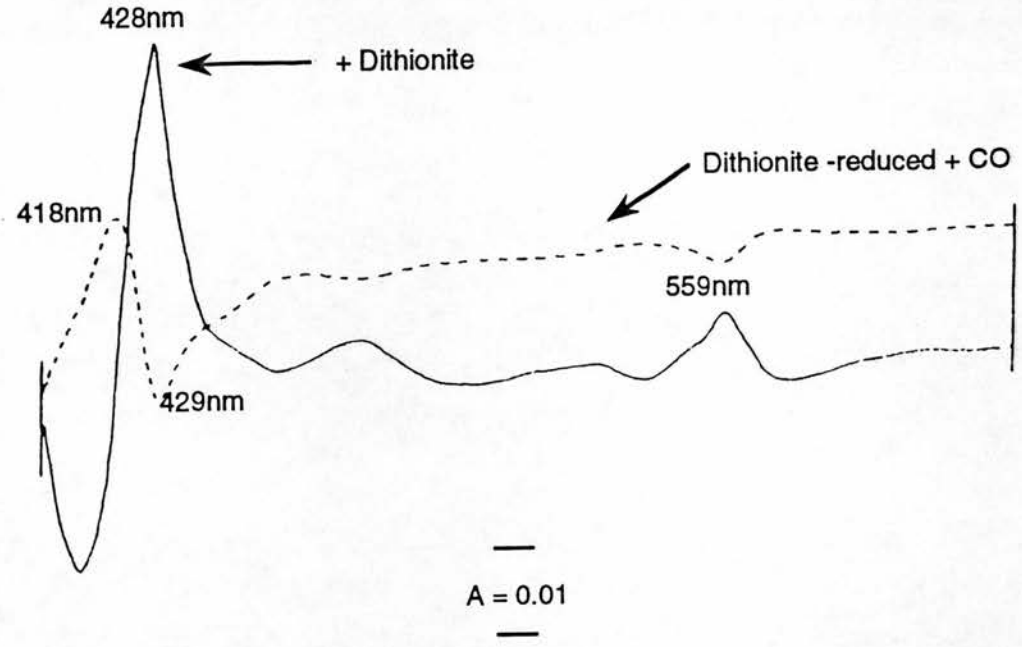

Figure 5.1 Spectral analyses of cytochrome b_{558} from the plasma membrane of human neutrophils. ——— shows the dithionite-reduced minus oxidized spectrum; - - - - - shows the (dithionite-reduced)+CO minus dithionite reduced spectrum.

Another experimental approach adopted in these studies were Western blot analyses of human sperm proteins using polyclonal antibodies raised against components of the phagocytic leucocyte NADPH oxidase. Western blotting or immuno-blotting, as it is sometimes referred to (Renart *et al*, 1979; Towbin *et al*, 1979), is a variation on the Southern blotting technique developed by Southern in 1975 for analysing DNA fragments separated on agarose gels, by transferring these fragments to a nitrocellulose membrane where they were probed by RNA or cDNA (Southern, 1975). After the development of Southern

blotting, a technique was developed for the blotting of RNA onto nitrocellulose membranes, and the subsequent probing of the separated RNA molecules with nucleic acid probes (Alwine *et al*, 1977). This technique was rather unimaginatively termed 'Northern Blotting', and hence a trend was set for the naming of blotting techniques, with the subsequently developed method of protein blotting and immuno-detection of the separated molecules, being termed 'Western Blotting'. Thus, Western blotting involves the transfer of proteins, onto nitrocellulose or other membrane supports, which have been separated by polyacrylamide gel electrophoresis, rather than the transfer of DNA or RNA fragments. Western blotting has become a widely used technique for the identification of protein antigens recognised by antibodies (Stott, 1989), and it is an extremely powerful technique, which has revolutionised the detection of antigen and antibodies (Poxton, 1990). The technique combines the powerful resolution of gel electrophoresis, with the specificity of immunochemical detection. In this chapter, Western blot experiments will be described that investigate the possible presence of leucocyte NADPH oxidase components in human spermatozoa. These studies were conducted using a panel of polyclonal antibodies, raised in rabbit, against individual components of the human phagocytic leucocyte NADPH oxidase. In this chapter Western blots were constructed employing polyclonal antibodies raised against the α and β subunits of the cytochrome b_{558}, and against $p47^{phox}$ and $p67^{phox}$.

Thus, with the above, numerous and multi-directional, approaches to pursue in an attempt to identify cellular components that may be involved in ROS generation by human spermatozoa, it is hoped that the data obtained will give us some indication as to the identity of the molecules involved in human spermatozoa ROS generation, and so finally clarify the situation as to whether or not human spermatozoa contain a NADPH oxidase complex, which is closely related to, or the same as, that of phagocytic leucocytes. The

experiments described in this chapter, therefore set out to determine whether the NADPH oxidase activities expressed by human spermatozoa and phagocytic leucocytes are similar in terms of the cellular mechanisms controlling their activity and the biochemical properties of their component parts.

5.2 Materials and methods

5.2.1 Cell preparation

Human semen samples were prepared as previously described and only the resulting spermatozoa suspensions that were free of leucocyte contamination, used in any of the following experiments. For luminometry, cells were suspended in BWW supplemented with albumin, when luminol-dependent chemiluminescence was employed to monitor ROS generation, whilst BWW supplemented with PVA was the sperm suspension medium used when lucigenin-dependent chemiluminescence was the ROS detection technique employed.

5.2.2 ROS generation

All luminometer-based experiments were performed at least three times, on populations of spermatozoa from different semen donors, and the results expressed as independent, representative, longitudinal analyses, and as the means of the repeated measurements, with analysis of variance (ANOVA) statistical analyses being performed to indicate any statistical significance.

In all experiments, where PMA was used as the stimulus of ROS generation, the very sensitive luminol-dependent assay of chemiluminescence was employed to monitor any ROS generated. The less sensitive, but NADPH compatible, lucigenin-dependent assay of chemiluminescence was employed in experiments which were based on the ability of exogenous NADPH to

induce ROS generation by human spermatozoa. Unless otherwise stated the sperm suspensions used were at a concentration of $10x10^6$ cells/ml.

5.2.3 Effect of PMA on NADPH-induced ROS generation

Spermatozoa were stimulated to generate the superoxide anion as described in Chapter 3 of this thesis, with 500µM NADPH. Superoxide anion generation was monitored by lucigenin-dependent chemiluminescence for around 15 minutes and then 4µl of a stock solution of PMA at a concentration of 10µM in 1% DMSO in BWW (resulting in a final working concentration of PMA of 100nM),were added. Subsequent chemiluminescence was monitored for a further 45 minutes and the results analyzed as follows. The integrated chemiluminescent signals over two five minute periods were calculated, i.e. immediately before and after PMA addition. Any PMA-dependent change in chemiluminescence was then calculated by subtracting the number of chemiluminescent counts calculated for the 5 minute period prior to PMA addition, from the total number of chemiluminescent counts calculated in the five minute period after PMA addition. The results were expressed as the mean chemiluminescence of three separate experiments (counts /5 mins). The results have also been presented as traces of representative, individual experiments.

5.2.3 Effect of PKC inhibitors on ROS generation

Prior to stimulation with PMA, 400µl of spermatozoa in BWW supplemented with albuminar, were incubated with 4µl of staurosporine (Sigma) at a concentration of 100µM in DMSO, resulting in a final working concentration of staurosporine of 1µM. Alternately, 45µl of H7 (Sigma) was added to the sperm suspensions at concentrations of 100µM, 50µM, 25µM, and 10µM in PBS, resulting in final working concentrations of H7 of 10µM, 5µM, 2.5µM, and 1µM respectively. As controls, spermatozoa suspensions had either 4µl of DMSO or

45µl PBS added. ROS generation was monitored by luminol-dependent chemiluminescence, and once a steady basal signal had been obtained, usually after about 10 minutes, 4µl of a stock solution of PMA at a concentration of 10µM in 1% DMSO in BWW (resulting in a final working concentration of PMA of 100nM), were added. Subsequent chemiluminescence was then monitored for approximately 20 minutes, and the results analyzed as above. The results have also been presented as traces of representative, individual experiments, in order to demonstrate the detailed kinetics of chemiluminescence response observed.

5.2.5 Effect of Okadaic acid on ROS generation

Okadaic acid was purchased from Calbiochem (Calbiochem) and was resuspended in DMSO at a concentration of 100µM. This stock solution was aliquotted and stored at -20°C until use. In all experiments okadaic acid was added to the spermatozoa suspensions prior to addition of the stimulator of ROS generation, in this case before the addition of PMA. 4µl of okadaic acid was added to 400µl spermatozoa at concentrations of 10µM, 1µM, 100nM and 10nM, all in DMSO, yielding final working concentrations of okadaic acid of 100nM, 10nM, 1nM. and 0.1nM respectively. ROS generation was then monitored by luminol-dependent chemiluminescence until a steady basal signal was obtained. At this point 4µl of PMA, at a concentration of 10µM in DMSO (final working concentration 100nM) was added. The resulting chemiluminescent signal was monitored for around twenty minutes, and the results were analyzed as described before.

5.2.6 Effect of diphenylene iodonium (DPI) on ROS generation

DPI was a generous gift from Professor Owen Jones of the Department of Biochemistry at the University of Bristol. The compound was synthesised in Bristol according to the method of Collette *et al* (1956). The DPI was dissolved

in 10% DMSO by sonification, in a Transonic T310 sonicator (Camlab Limited, Cambridge, England), to give a 10mM stock solution. This stock solution was aliquotted into small volumes and stored at -20°C until it was used. Before use, the DPI was defrosted and diluted to the appropriate concentration with 10% DMSO. In all experiments, DPI was added to the spermatozoa before the addition of PMA or NADPH. Briefly, 4µl of DPI stocks at concentrations of 1µM, 10µM, 100µM, 500µM, 1mM, 2.5mM, 5.0mM, or 10mM were added to 400µl aliquots of spermatozoa resulting in final working concentrations of DPI of 10nM, 100nM, 1µM, 5µM, 10µM, 25µM, 50µM, and 100µM respectively. Control aliquots of spermatozoa had 4µl of 10% DMSO added to them in place of the DPI. ROS generation by the suspensions of spermatozoa was monitored for approximately ten minutes, or until a steady basal signal had been achieved, and then either 4µl of PMA, at a concentration of 10µM in DMSO (final working concentration 100nM), or 45µl of NADPH, at a concentration of 5mM in PBS (final working concentration of NADPH 500µM), was added. The resulting chemiluminescent signal was monitored for around twenty minutes. The results were analyzed as described earlier.

5.2.7 Effect of quinacrine on ROS generation

Quinacrine was purchased from Sigma, and dissolved in DMSO to give a stock solution of 10mM. This stock was made fresh daily and then diluted with DMSO to give the required working stock solutions. In all experiments, quinacrine was added to the spermatozoa before the addition of PMA or NADPH. Briefly, 4µl of quinacrine at concentrations of 250µM, 500µM, 1mM, 5mM, 7.5mM, or 10mM were added to 400µl aliquots of spermatozoa resulting in final working concentrations of quinacrine of 2.5µM, 5µM, 10µM, 50µM, 75µM, and 100µM, respectively. Control aliquots of spermatozoa had 4µl of DMSO added to them in place of the quinacrine. ROS generation by the spermatozoa was then monitored for approximately ten minutes, or until a

steady basal signal had been achieved, and then either 4µl of PMA, at a concentration of 10µM in DMSO (final working concentration 100nM), or 45µl of NADPH, at a concentration of 5mM in PBS added (final working concentration of NADPH 500µM) was added. The resulting chemiluminescent signal was monitored for around twenty minutes. The results were analyzed as described earlier.

5.2.8 Plasma membrane isolation

Sperm plasma membranes were isolated according to the method of Gillis *et al* (1978) as modified by Aitken *et al* (1987). Leucocyte free sperm samples were used in all instances. The samples were centrifuged, 500*g* for 5 minutes, and the supernatants removed. The sperm pellets were resuspended in 1mM EDTA (pH5.0) containing a protease inhibitor, 1mM phenylmethyl sulphonyl fluoride (PMSF) (Sigma). The sperm membranes were detached by sonification using a Soniprep ultrasonicator (MSE, Crawley, Sussex, UK), using 3 x 10 second bursts with a probe amplitude of 30µm. The sperm preparations were kept on ice between sonification bursts to minimize any undesired heating effects. The ultrasonicated suspensions were centrifuged at 500*g* for 5 minutes to pellet any remaining intact and de-membranated spermatozoa, and the supernatant from this stage was then centrifuged at 3,000*g* for 5 minutes to remove any residual cells present. The supernatant was then centrifuged at 100,000*g* for 1 hour in an ultracentrifuge (Sorvall OTD-50; Du Pont Instruments, Stevenage, Herts, UK). The crude membrane pellet obtained at this point was either resuspended in PBS and used in this form (designated the crude membrane preparation), or resuspended in 0.25M sucrose and carefully layered onto a discontinuous sucrose gradient (1.57M, 1.3M, 1.0M sucrose) and centrifuged at 100,000*g* for 2 hours, in order to isolate the sperm plasma membranes. The plasma membrane fraction was collected as a discreet layer at the 1.0-1.3M interface, diluted in PBS and centrifuged at 100,000*g* for 1 hour

to pellet the membranes. The plasma membrane pellets were resuspended in small, known volumes of PBS, an aliquot taken for estimation of protein content (BCA method as described in Chapter 3), and the remainder frozen at -20°C until required.

5.2.9 Spectral analyses of spermatozoa plasma membrane cytochrome content

All the spectral analyses were kindly performed by Dr. John Hancock, of the Department of Biochemistry, at the University of Bristol, UK, and his contribution to this work is gratefully acknowledged. The sperm plasma membranes and crude sperm membranes were prepared in Edinburgh, and transported to Bristol, on dry ice, by overnight courier, where they were immediately subjected to spectral analyses, as described below.

Briefly, reduced minus air oxidized difference spectra, and reduced + carbon monoxide (CO) minus reduced difference spectra of the sperm plasma membranes and crude membranes were recorded with a custom-built, rapid-scanning, split beam spectrophotometer, at 77°K, according to the method of Cross *et al* (1981). Reduction was performed anaerobically, in PBS by the addition of small amounts of sodium dithionite solution or NADPH (25µM and 250µM). The final volume in the cuvettes, in all instances, was 400µl.

Reduced -minus-oxidised difference spectra

In this instance, the reference cuvette contained an identical membrane suspension as the sample cuvette, without any added reductants, i.e. the air-oxidized sample. The absorbance of the reduced membrane preparations, i.e. those treated with sodium dithionite or NADPH, were measured, between 400nm and 650nm, against the oxidised membrane preparations (the reference samples), at 77°K, and resulting difference spectra recorded.

Dithionite reduced + CO-minus-dithionite reduced difference spectra

In this instance, the reference cuvette contained the dithionite reduced membrane preparations, whilst the sample cuvette contained the dithionite reduced + CO membrane preparations. Dithionite reduced + CO membrane preparations were prepared by saturating dithionite reduced membrane preparations with CO gas (BDH), in the spectrophotometer cuvette. These were then stoppered tightly with Teflon® caps and placed in the sample position of the split beam spectrophotometer. The absorbance of the dithionite reduced + CO membrane preparations were then measured, between 400nm and 650nm, against that of the dithionite reduced preparations (the reference samples), at 77°K, and the resulting difference spectra recorded.

5.2.10 Western blotting

SDS-solubilized sperm proteins were subjected to SDS-PAGE under reducing and non-reducing conditions, as previously described, along with purified NADPH oxidase components of human polymorphonuclear leucocytes. The SDS-solubilized protein from $5x10^6$ spermatozoa was used/lane. The positive control NADPH oxidase proteins were used in these experiments at concentrations recommended by Professor Tony Segal, Department of Medicine, University College, London, who generously provided these samples for analyses. The molecular weight standards used were pre-stained protein molecular weight markers from BioRad, the contents of which were described in Chapter 3 of this thesis. 10µl of the standards were used per lane and were mixed 1:1 with sample buffer and boiled for 5 minutes.

After electrophoresis, the proteins were transferred from the gel on to Hybond C™- super, supported nitrocellulose membranes (Amersham), by semi-dry blotting, as described in Chapter 3 of this thesis. Post-blotting, the nitrocellulose membranes were incubated with primary and secondary antibodies, again as described before. The primary antibodies used were

polyclonal antibodies raised in rabbit, against individual components of the human leucocyte NADPH oxidase, and were very generously supplied by Professor Tony Segal, of the Department of Medicine, University College London, England. Two antibodies against the cytochrome b_{558} of the NADPH oxidase were used, i.e. one against the large, β-subunit and one against the small, α-subunit. These two antibodies were used at the recommended dilution of 1:1000 and also at 1:500. Antibodies against the $p47^{phox}$ and $p67^{phox}$ cytosolic components of the oxidase were also used. The anti-p47 antibody was used at dilutions of 1:400 and 1:200, and the affinity purified, anti-p67 antibody was used at dilution's of 1:1000 and 1:500. The membranes were incubated in these primary antibody solutions overnight at 4°C, and then subjected to the usual washing procedure. All the blots were then incubated with secondary antibody for 1 hour at room temperature. The secondary antibody used was a horseradish peroxidase conjugated polyclonal antibody, raised in donkey, against rabbit IgG (Amersham). This antibody was used at a dilution of 1:6000. After incubation with the secondary antibody, the nitrocellulose membranes were again subjected to the usual washing procedure and then processed for protein detection using the enhanced chemiluminescent Western blotting detection kit from Amersham.

5.3 Results

5.3.1 Effect of PMA on NADPH-induced ROS generation

Addition of 100nM PMA to sperm suspensions had no effect on NADPH-induced superoxide anion generation by these cells as monitored by lucigenin-dependent chemiluminescence (Figure 5.2a and b).

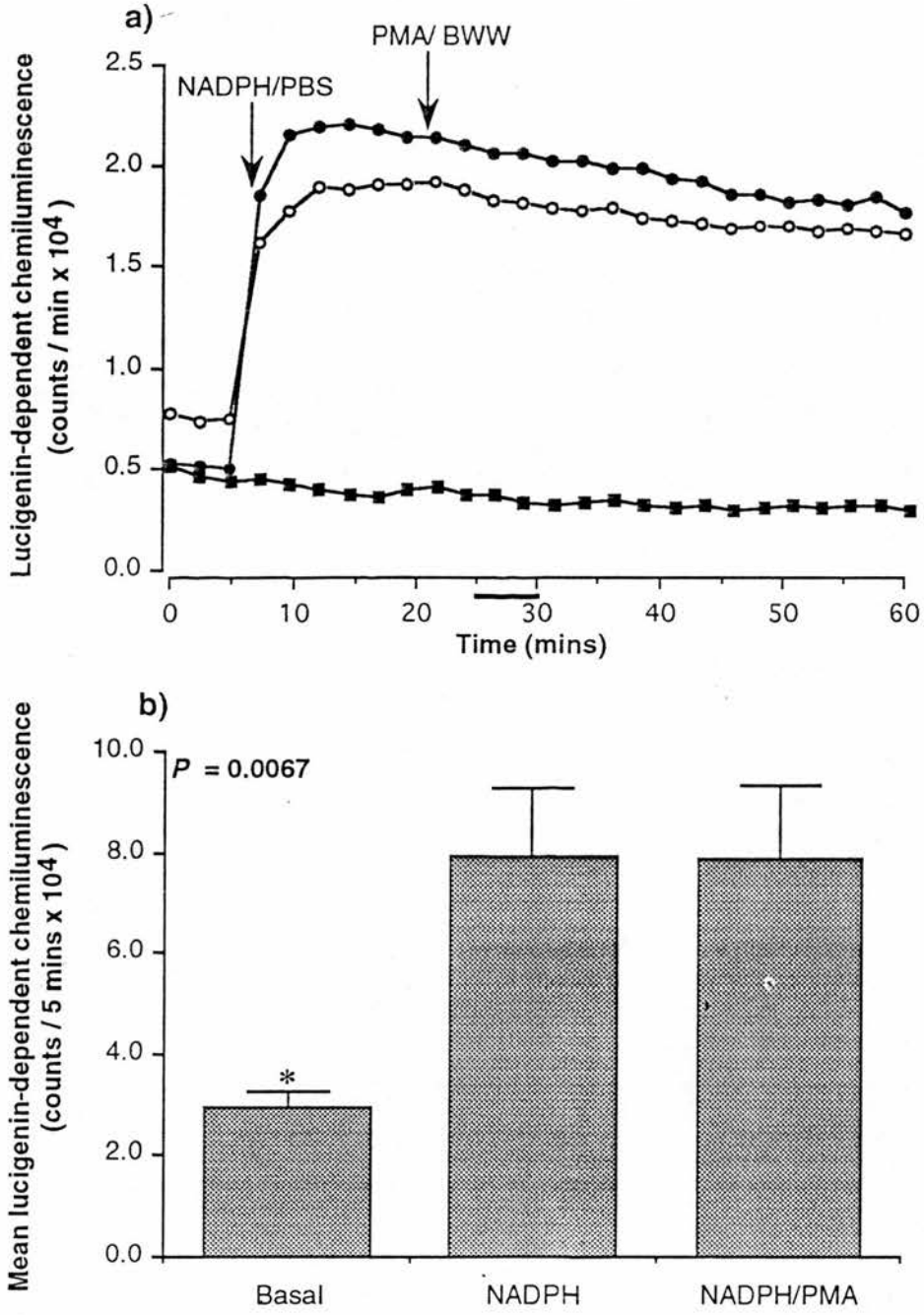

Figure 5.2 Effect of PMA on NADPH-induced, lucigenin-dependent chemiluminescence by human spermatozoa. Cell suspensions, except the control, were stimulated with NADPH (500μM) and then, 10 minutes later PMA (100nM) or BWW was added. **a)** is an individual trace, representative of 4 separate experiments, whilst, **b)** shows the means ±S.E. of the 4 separate experiments. Basal lucigenin-dependent chemiluminescence was significantly lower than that stimulated with NADPH, $*P < 0.05$, but PMA did not have any significant effect on the NADPH response.

Treatments: Control =—■— ; NADPH alone =—●— ;
NADPH+ PMA =—○— .

ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

5.3.2 Effect of inhibitors of protein kinase C on PMA induced ROS generation by human spermatozoa

Both staurosporine and H7 inhibited ROS generation by human spermatozoa in response to PMA. The one concentration of staurosporine employed, 1μM, succeeded in significantly inhibiting PMA-induced ROS generation to approximately 13% of the control response to PMA, $P<0.05$ (Figure 5.3). H7, a less specific inhibitor of PKC also inhibited PMA induced ROS generation though this inhibition was only statistically significant at the highest concentration used, i.e. 100μM, $P<0.05$ (Figure 5.4). The lack of statistical significance at lower concentrations may be due to the large donor to donor variation observed in the extent of H7-dependent inhibition of ROS generation. The individual trace (Figure 5.3a) shows the consistent pattern of H7-dependent inhibition.

5.3.3 Effect of Okadaic acid on ROS generation

At okadaic acid concentrations of 0.1nM 1.0nM, and 100nM, no significant effect on PMA induced ROS generation was observed, but at a concentration of 10nM, a significant increase in ROS generation in response to PMA was observed, $P<0.05$ (Figure 5.5). Inclusion of this concentration of okadaic acid with the spermatozoa resulted in up to a 3 fold increase in the response to PMA.

5.3.4 Effect of DPI on ROS generation

Addition of DPI to suspensions of spermatozoa prior, to the addition of either stimulus of ROS generation, i.e. PMA or NADPH, resulted in a significant, dose dependent, inhibition of ROS generation. When spermatozoa were stimulated to generate ROS with PMA, significant inhibition was achieved with 10nM DPI ($P<0.05$), whilst almost complete inhibition was achieved with 1μM and 10μM ($P<0.05$) (Figure 5.6). When spermatozoa were stimulated to

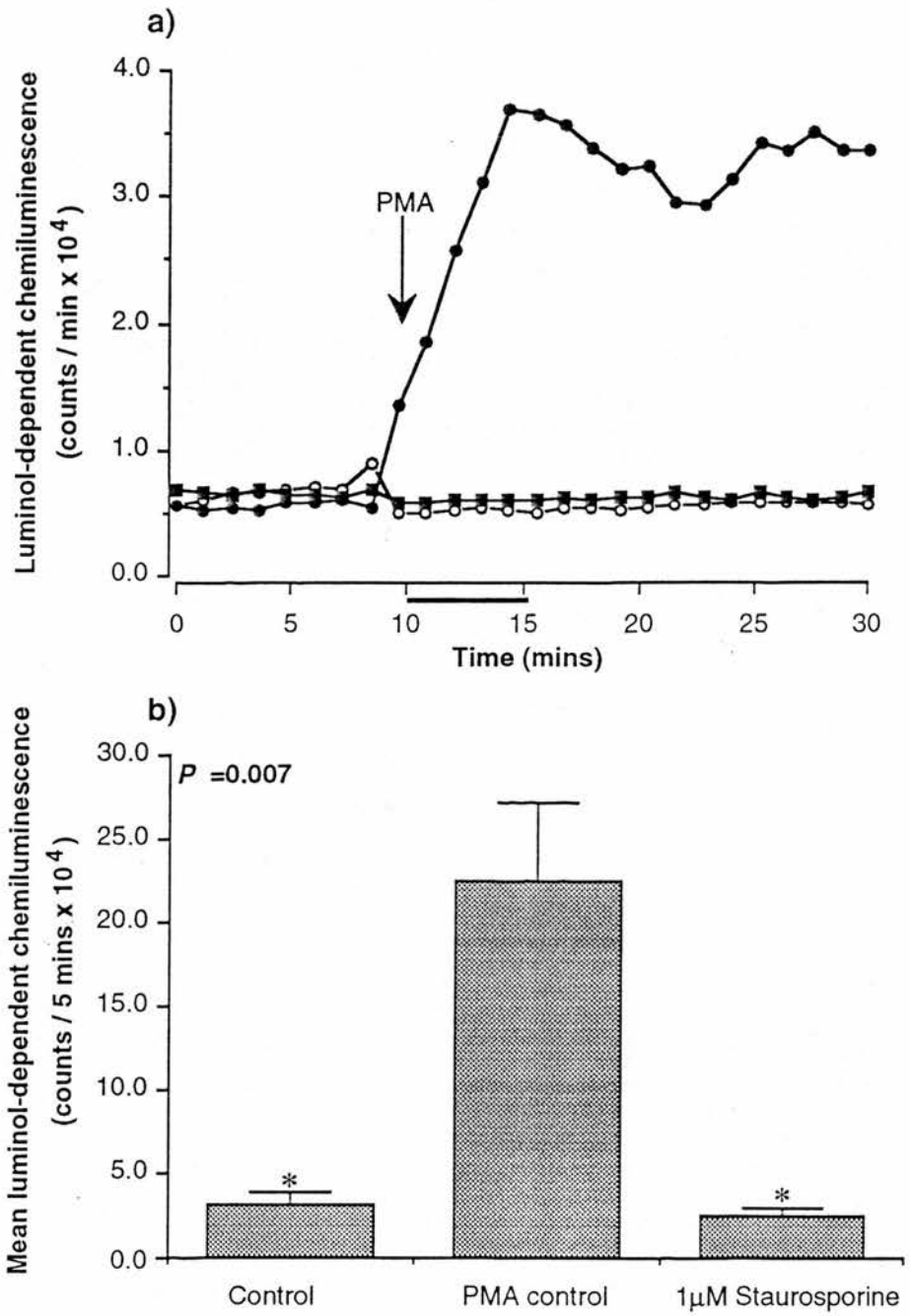

Figure 5.3 Effect of staurosporine on PMA induced, HRP-enhanced, luminol-dependent chemiluminescence. Cell suspensions were stimulated with PMA (100nM) in the presence or absence of staurosporine (1μM). **a)** is an individual trace, representative of 3 separate experiments, whilst **b)** shows the means± S.E. of the 3 separate experiments. Staurosporine significantly inhibited the PMA response, down to basal chemiluminescence levels, $*P < 0.05$. Overall, the levels of observedchemiluminescence were significantly different ($P = 0.007$).
Treatments: control (no PMA)= —■— ; PMA control= —●— ; and PMA/staurosporine= —○— .
ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

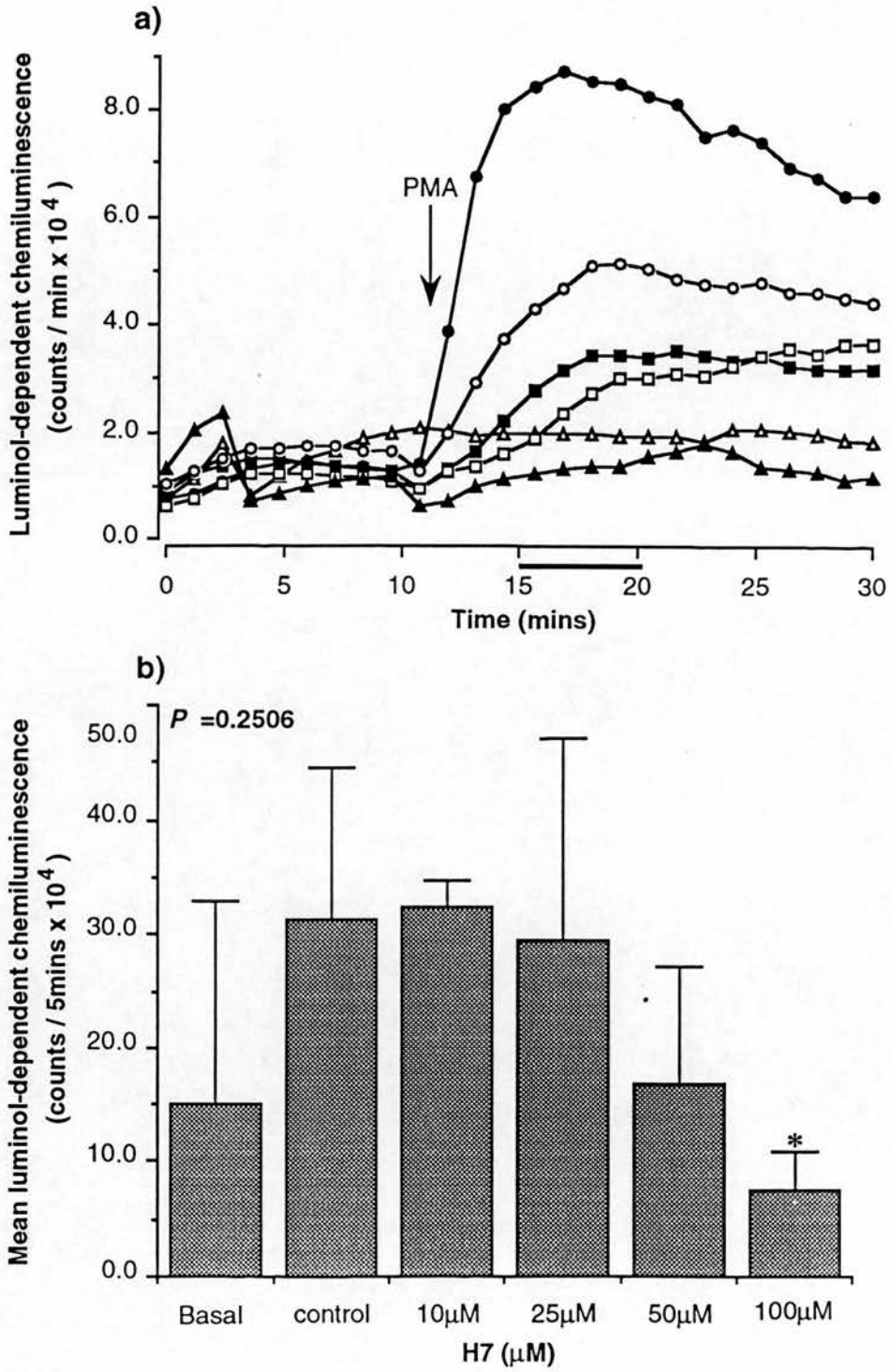

Figure 5.4 Effect of H7 on PMA-induced, HRP-enhanced, luminol-dependent chemiluminescence. Human sperm suspensions were stimulated with PMA (100nM) in the presence of various concentrations of H7. **a)** is an individual trace, representative of 3 separate experiments, whilst **b)** shows the means± S.E. of the 3 separate experiments. 100μM H7 significantly inhibited the PMA reponse, $*P<0.05$.
Control (no PMA) = —△— ; PMA control= —●— ; 10μM H7= —■— ; 25μM H7=—○— ; 50μM H7= —□— ; 100μM H7= —▲—.

ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

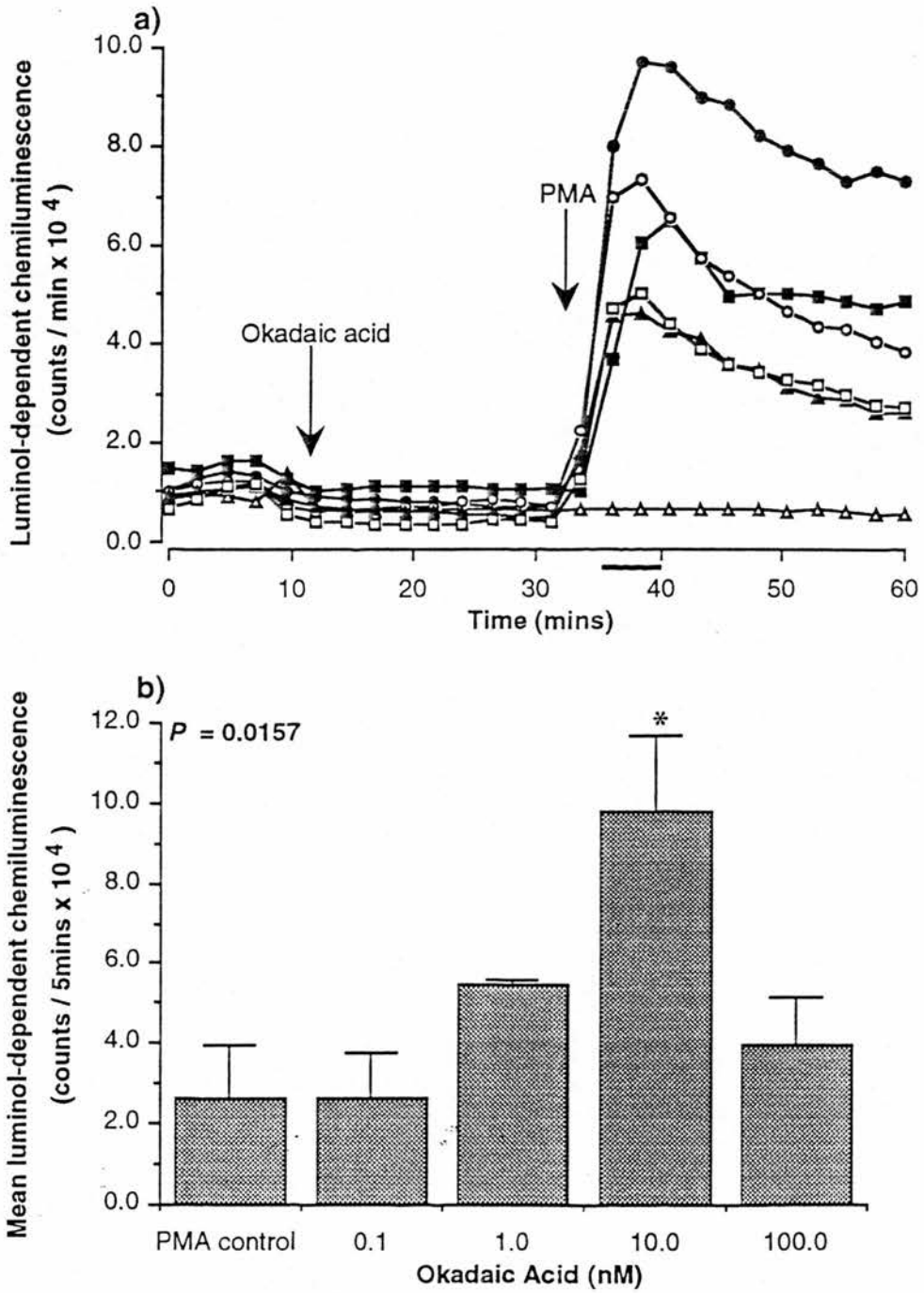

Figure 5.5 Effect of okadaic acid (OA), on PMA-induced, HRP-enhanced, luminol-dependent chemiluminescence. Cell suspensions were stimulated with PMA (100nM), in the presence of various concentrations of OA. **a)** is an individual trace, representative of 7 separate experiments, whilst **b)** shows the mean ±S.E. of the 7 experiments. 10nM OA significantly stimulated the PMA response, $*P < 0.05$. Overall, the responses were significantly different from one another ($P = 0.0157$).
Treatments: Control (no PMA)= —△—; PMA control= —■—;
0.1nM OA= —▲—; 1.0nM OA= —○—; 10.0nM OA= —●—;
100nM OA= —□—.
ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

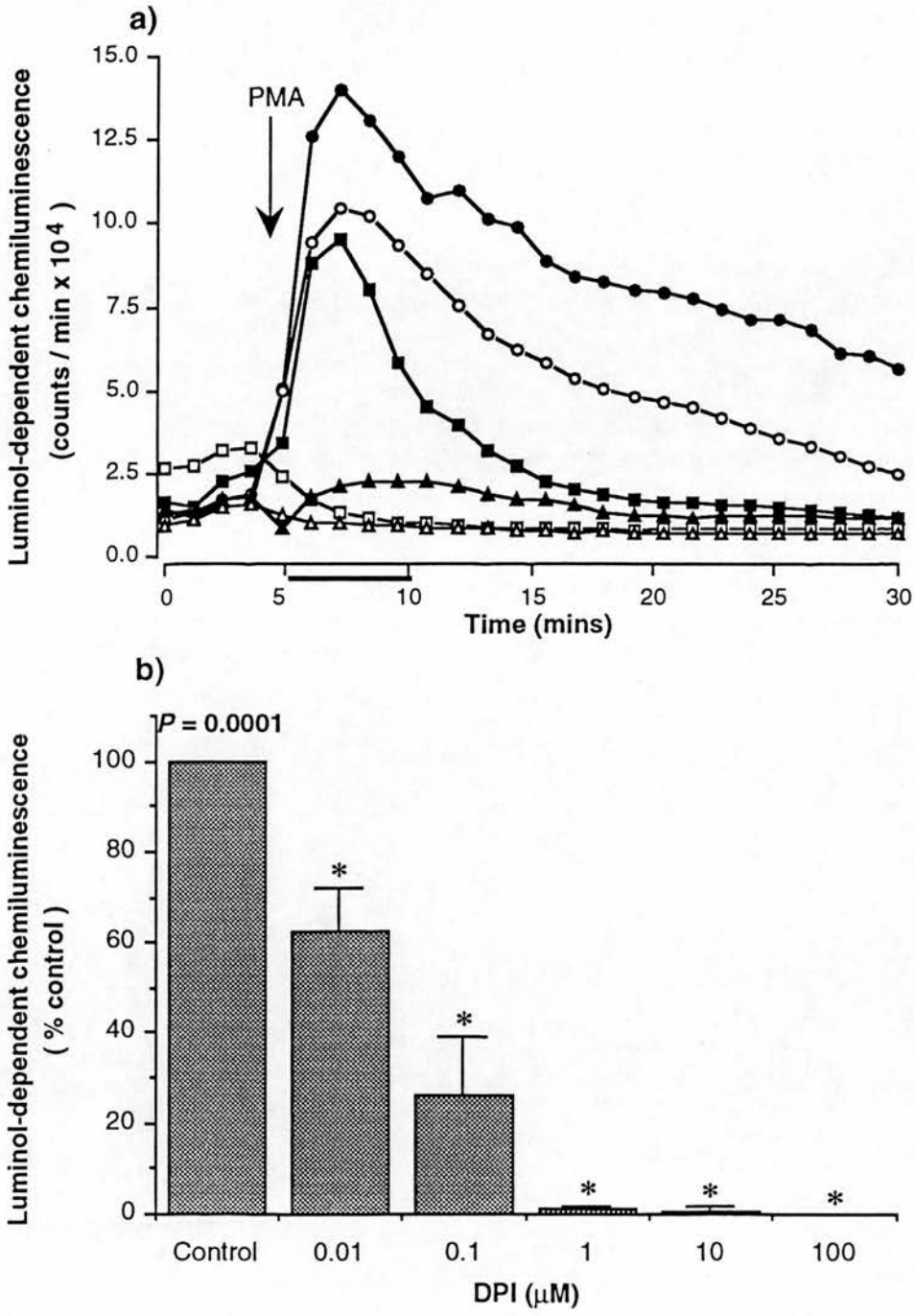

Figure 5.6 Inhibitory effect of diphenylene iodonium (DPI) on PMA-induced, HRP-enhanced, luminol-dependent chemiluminescence. Cell supensions were stimulated with PMA (100nM), in the presence of various concentrations of DPI. **a)** is a representative trace of 4 separate experiments, whilst **b)** shows the means ±S.E. of the 4 experiments, expressed as a % of the control (i.e. no DPI). DPI significantly inhibited the PMA response at all concentrations tested, $^*P < 0.05$. Overall, the various responses to PMA were significantly different from one another ($P = 0.0001$).

Treatments: control= —●—; 10nM DPI= —○—; 100nM DPI=—■—; 1μM DPI= —□—; 10μM DPI= —▲— ; 100μM DPI=—△— .

ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

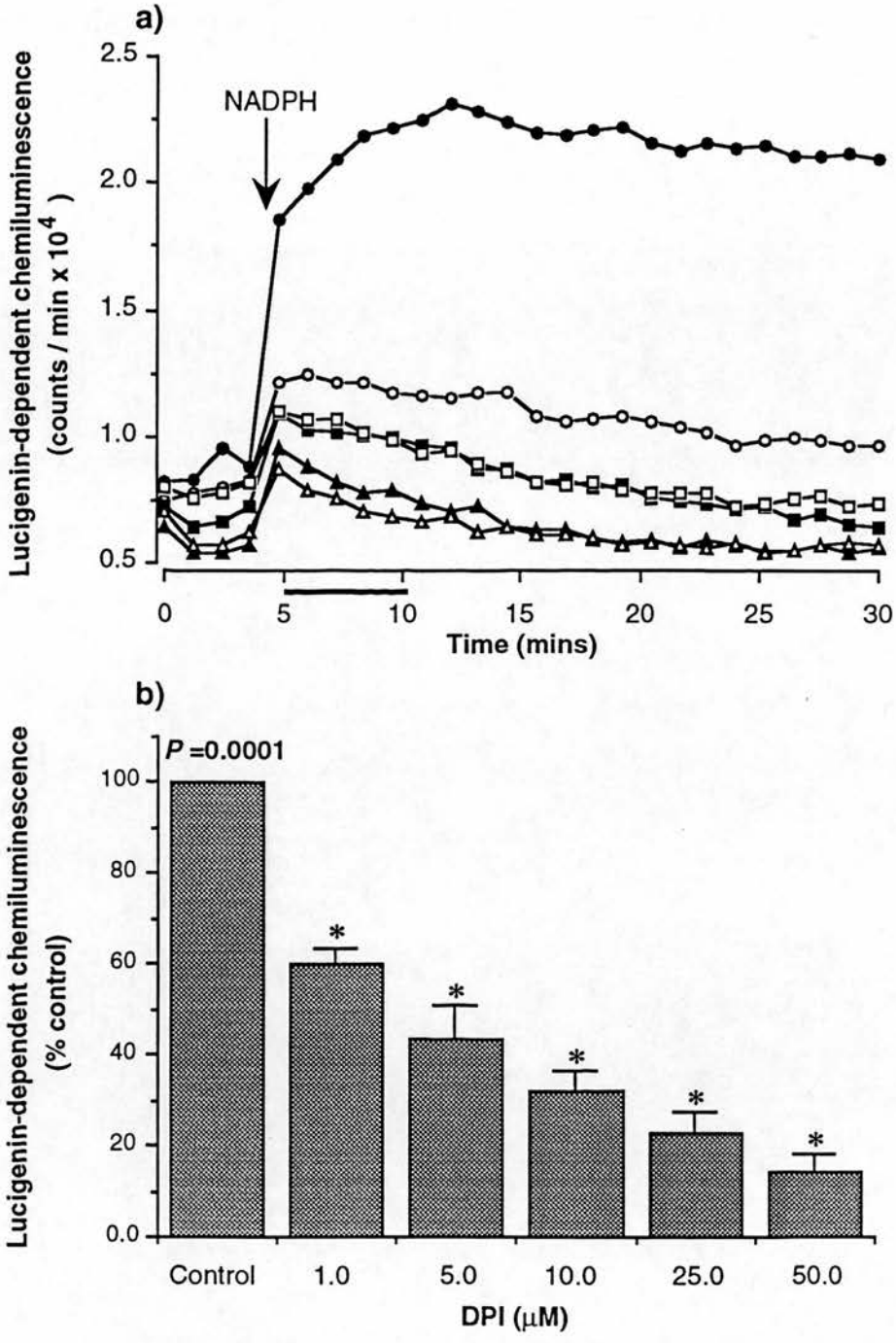

Figure 5.7 Inhibitory effect of DPI on NADPH-induced, lucigenin-dependent chemiluminescence. Cell suspensions were stimulated with NADPH (500μM), in the presence of various concentrations of DPI. **a)** is a representative trace of 7 separate experiments, whilst **b)** shows the means ±S.E. of the 7 experiments, expressed as a % of the control (i.e. no DPI). DPI significantly inhibited the NADPH response at all the concentrations tested, $*P < 0.05$. Overall, the various responses to NADPH were significantly different from one another ($P = 0.0001$).
Treatments: control=—●— ; 1.0μM DPI=—○— ; 5.0μM DPI=—■— ; 10.0μM DPI=—□—; 25.0μM DPI=—▲—; and 50.0μM DPI=—△— .

ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

generate ROS with exogenous NADPH, significant inhibition was observed with 1μM DPI ($P<0.05$), whilst the inhibition with 50μM DPI was very pronounced, ROS generation being inhibited to only around 20% of the control response ($P<0.05$) (Figure 5.7).

5.3.5 Effect of quinacrine on ROS generation

Quinacrine significantly inhibited ROS generation by human spermatozoa in response to PMA, in a dose-dependent fashion (Figure 5.8). Although the inhibition of ROS generation with quinacrine was only statistically significant at doses of 75μM and 100μM ($P<0.05$), all concentrations tested, except 10μM, inhibited PMA-induced ROS generation to some degree. At a concentration of 75μM, quinacrine brought the level of PMA-stimulated ROS generation down to less than 6% of the control, i.e. ROS generation in response to PMA with no prior addition of quinacrine, whilst 100μM completely abolished the response to PMA, with respect to ROS generation. However, at this concentration of quinacrine, the motility of the spermatozoa was compromised, as indicated by a decrease in the motility of the spermatozoa at the end of the experiment. However, the same pattern of results was not obtained when human spermatozoa were induced to generate ROS by the addition of exogenous NADPH (Figure 5.9). In this experimental situation, concentrations of quinacrine above 50μM completely immobilized sperm, probably indicating that they had been killed. At concentrations between 50μM and 5μM a significant reduction in the response to NADPH was observed ($P<0.05$), the reduction being approximately 40%. However, this reduction was not concentration-dependent, no significant difference being observed between the different quinacrine concentrations tested.

5.3.6 Spectral analyses of human sperm plasma membranes

The protein concentration of the sperm plasma membrane preparation was 4.38mg/ml and that of the crude sperm membrane preparation, 6.34mg/ml.

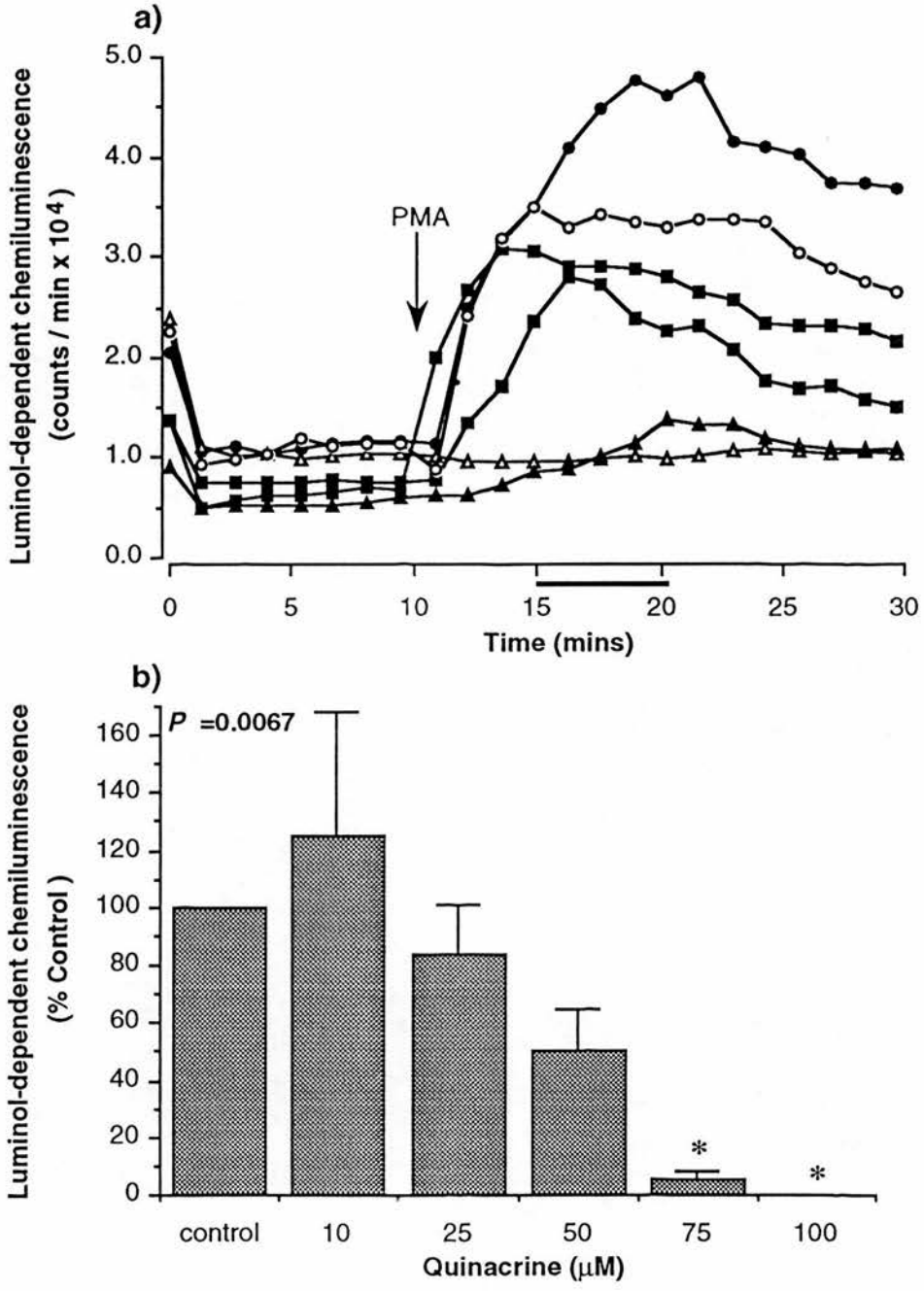

Figure 5.8 Effect of quinacrine on PMA-induced, HRP-enhanced, luminol-dependent chemiluminescence. Cell suspensions were stimulated with PMA (100nM), in the presence of various concentrations of quinacrine. **a)** is an individual trace, representative of 3 separate experiments, whilst **b)** shows the means ±S.E. of the 3 experiments, expressed as %s of the control (i.e. no quinacrine). Quinacrine, at concentrations of 75μM and 100μM, significantly inhibited the PMA response, $*P < 0.05$.

Control = —●— ; 10μM quinacrine= —○— ; 25μM quinacrine = —■—
50μM quinacrine = —■— ; 75μM quinacrine = —▲— ; and
100μM quinacrine = —△— .

ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

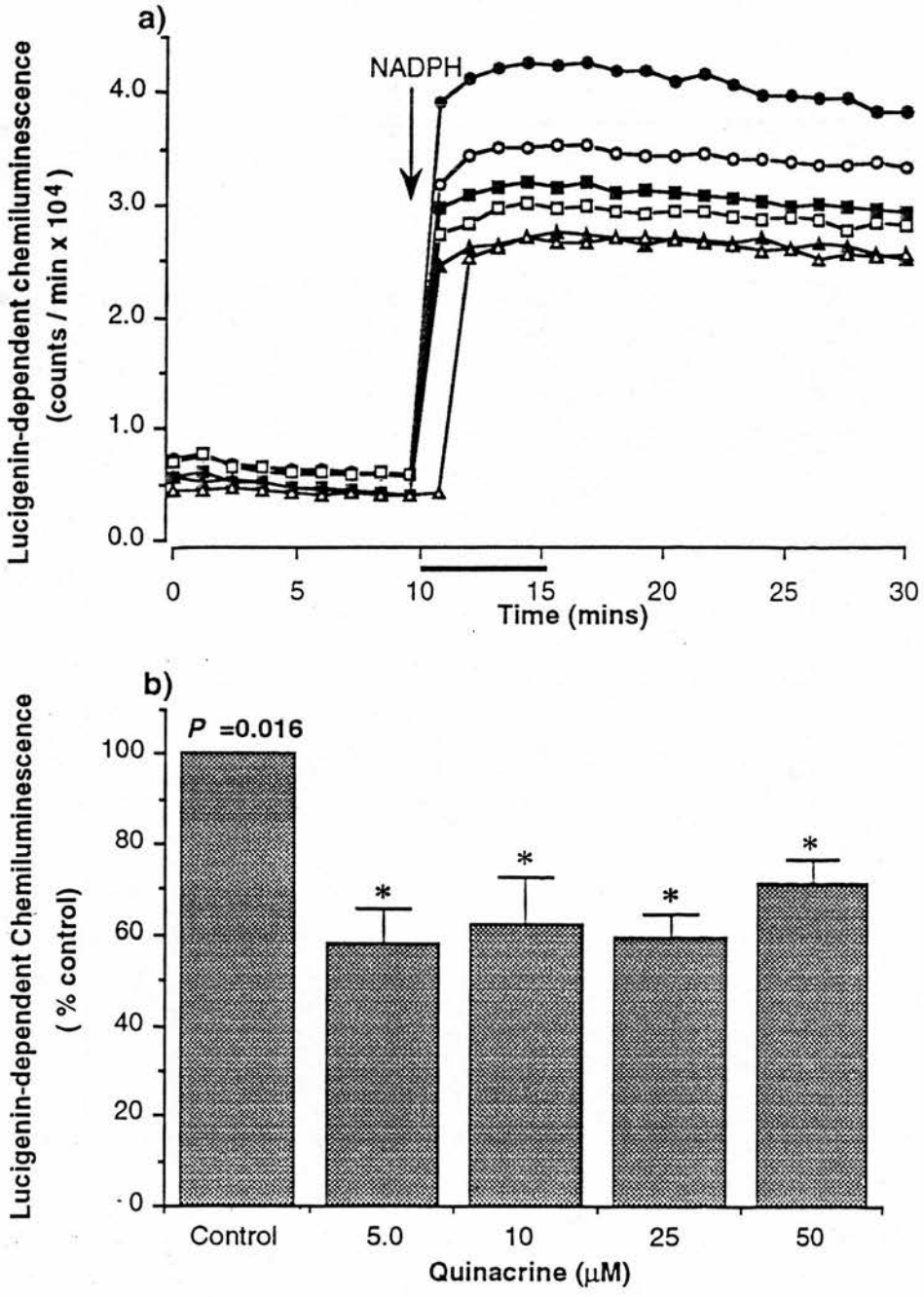

Figure 5.9 Effect of quinacrine on NADPH-induced, lucigenin-dependent chemiluminescence. Cell suspensions were stimulated with NADPH (500μM), in the presence of various concentrations of quinacrine. **a)** is an individual trace, representative of 3 separate experiments, whilst **b)** shows the means ±S.E. of the 3 experiments, expressed as %s of the control (i.e. no quinacrine). Quinacrine, at all concentrations tested, significantly inhibited the NADPH response, $*P < 0.05$.
Control =—●— ; 2.5μM quinacrine=—△— ; 5.0μM quinacrine =—▲—;
10.0μM quinacrine = —□— ; 25.0μM quinacrine =—■—; and
50.0μM quinacrine =—○— .
ROS generation was measured over the underlined, 5 minute interval indicated in graph a, to calculate the mean values of ROS generation shown in graph b. Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

650µg (1.625mg/ml) of protein were used in the plasma membrane based experiments and 1.2mg of protein (3.0mg/ml) used in the crude membrane preparation based experiments.

The traces in figure 5.10a show the NADPH or dithionite-reduced minus oxidised absorbance spectra of sperm plasma membranes, recorded at 77°K, from 400 to 650nm. The spectra of the sperm plasma membrane preparations failed to show any of the characteristic peaks typical of cytochrome b_{558}, i.e. peaks at 428nm and 558nm. No other cytochromes were apparent in these preparations either. Similarly, the traces in Figure 5.10b showing the NADPH or dithionite-reduced minus dithionite-reduced + CO absorbance spectra of sperm plasma membranes failed to show the presence of any cytochromes, low potential or otherwise. Spectral analysis of crude sperm membrane preparations also failed to show the presence of any cytochrome b_{558}, or other low potential cytochrome (Figure 5.11). However, these spectra did indicate the presence of the mitochondrial cytochrome c, indicated by a small peak at 549nm on the dithionite reduced minus oxidised peak (trace 3, Figure 5.11a). In figure 5.11a, trace 3, and Figure 5.11b there does seem to be a peak at around 428 and 418nm respectively, indicative of cytochrome b_{558}, but as there are no peaks (reduced minus oxidised) or troughs (reduced minus reduced + CO) at 558nm this cannot be taken to indicate the presence of cytochrome b_{558}.

5.3.7 Western blot analyses of human sperm proteins

The NADPH oxidase polyclonal antibodies were used to probe reduced and non-reduced sperm protein preparations and in each instance failed to cross react with the sperm protein (Figures 5.12 and 5.13). The antibodies cross reacted with the positive control protein preparations in all instances except the antibody raised against the large, ß-subunit of the cytochrome b_{558} (Figure 5.12b).

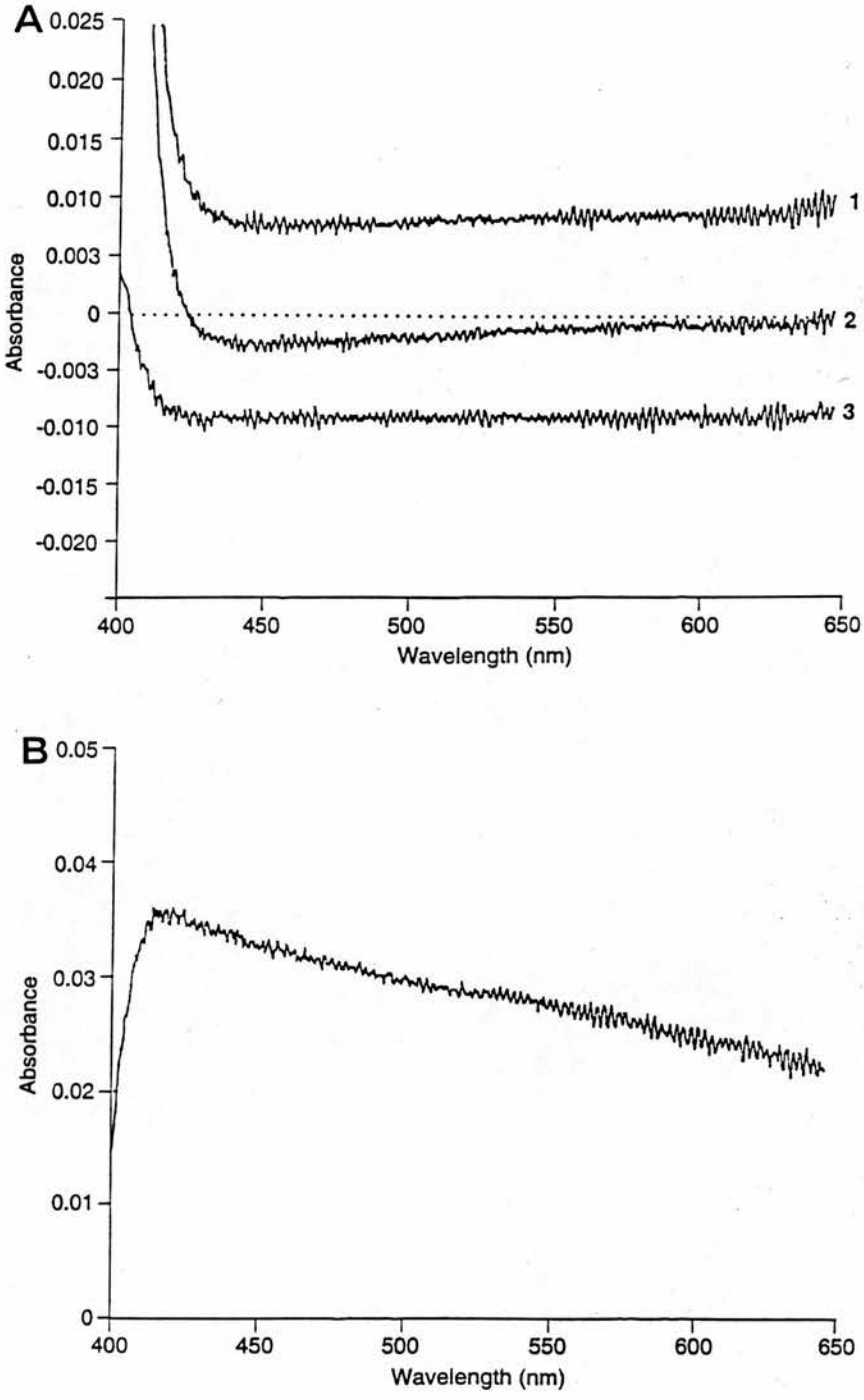

Figure 5.10 Spectral analyses of human sperm plasma membranes. Plot A shows the reduced minus oxidized absorbance spectra of the membrane preparations. Trace 1 shows dithionite reduced membranes; trace 2, membranes reduced with 250μM NADPH; and trace 3, membranes reduced with 25μM NADPH. Plot B shows the dithionite-reduced + CO minus dithionite-reduced absorbance spectrum of human sperm plasma membranes. Neither analysis revealed the presence of cytochrome.

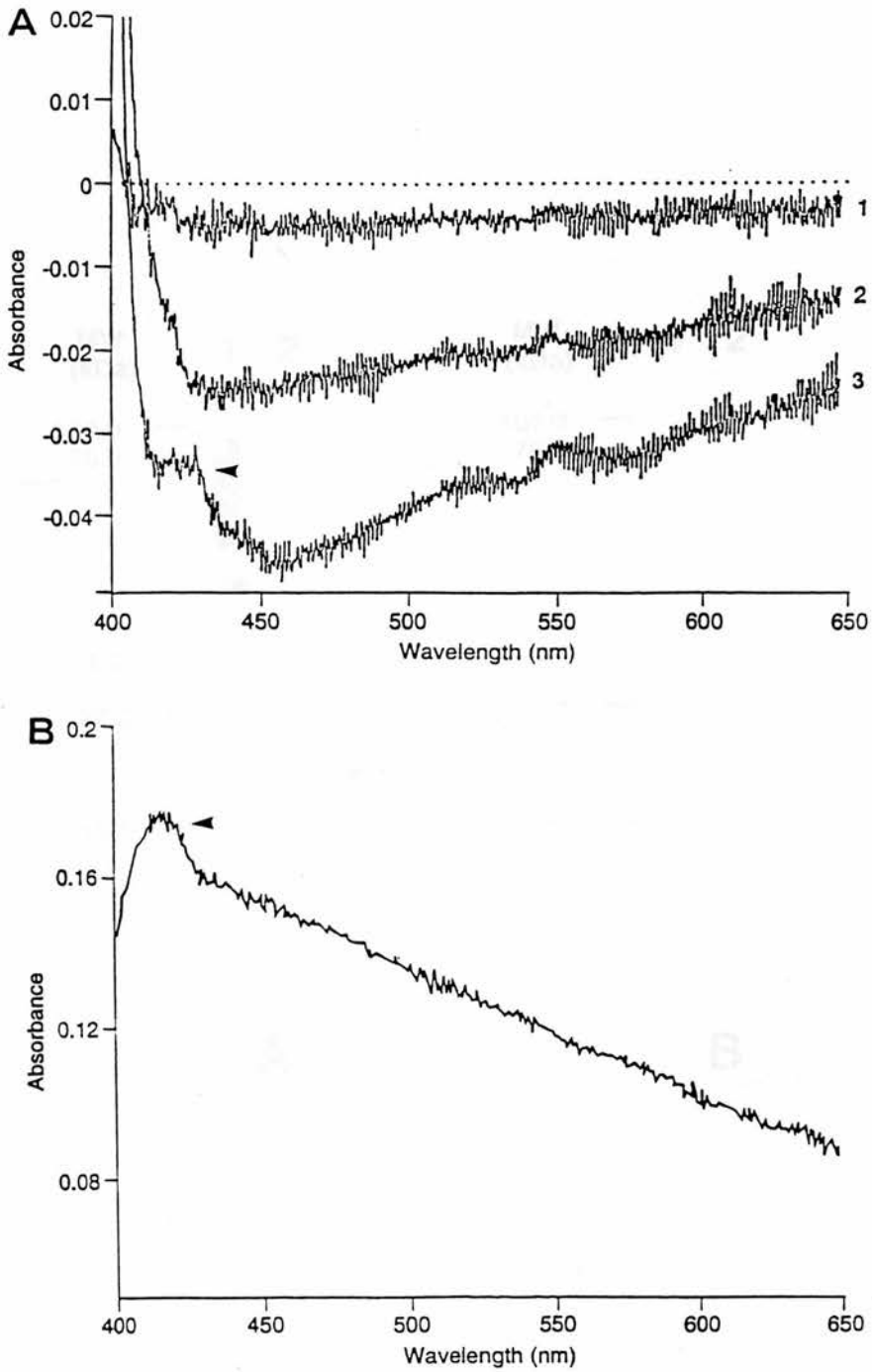

Figure 5.11 Spectral analyses of human sperm total membranes. Plot A shows the reduced minus oxidized absorbance spectra of the membrane preparations. Trace 1 shows membranes reduced with 25μM NADPH; trace 2, membranes reduced with 250μM NADPH; and trace 3, dithionite-reduced membranes. Plot B shows the dithionite-reduced + CO minus dithionite-reduced absorbance spectrum of human sperm membranes. Slight peaks are at 428nm and 418nm in trace 3, plot A and plot B respecitively, but these cannot be taken to indicate the presence of cytochrome b_{558}. However, plot A, trace3 does reveal the presence of mitochondrial cytochrome c at 549nm.

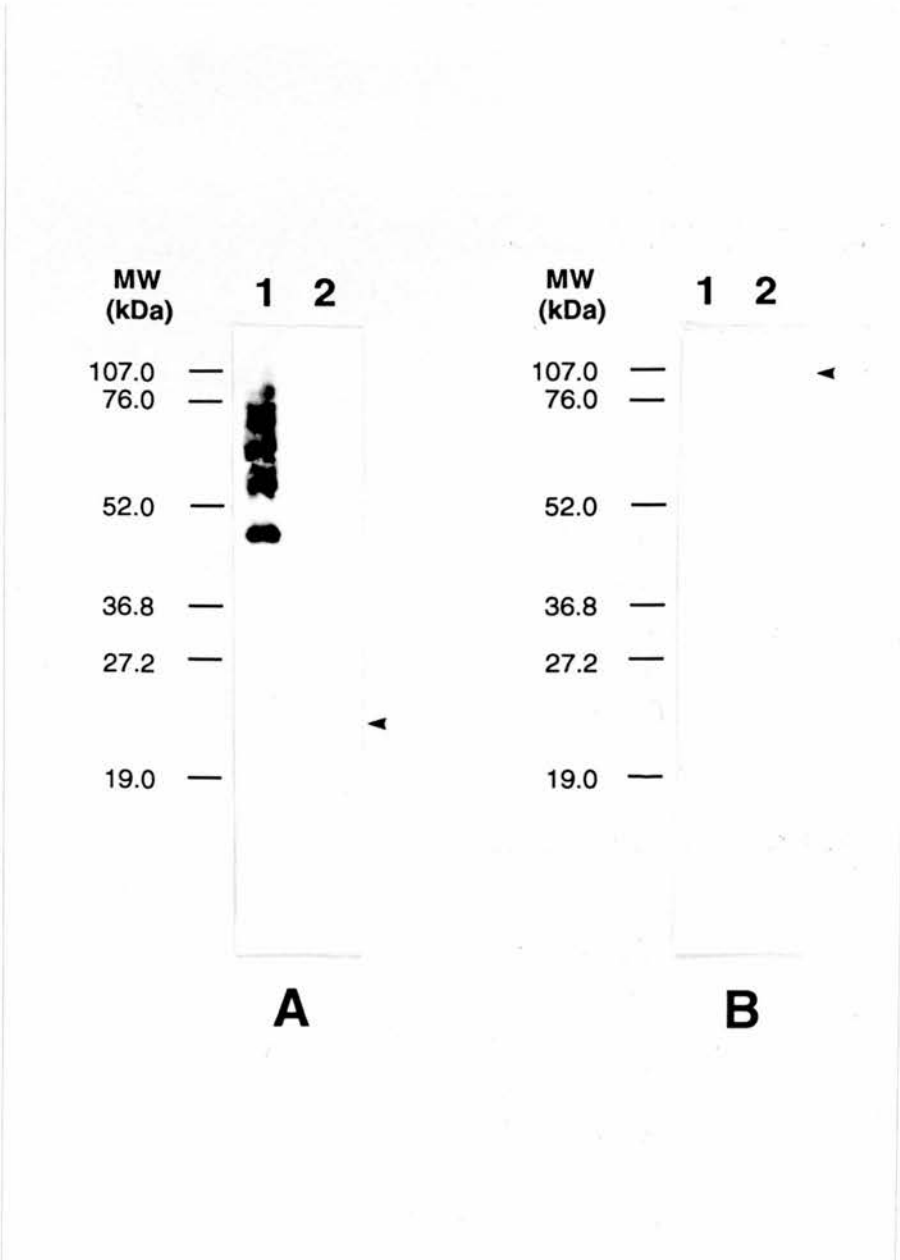

Figure 5.12 Western blots of SDS solubilized, human sperm protein, electrophoresed under reducing conditions. Panel A shows a blot probed with an antibody against the 22kDa, α subunit of cytochrome b_{558}. Lane 1, purified cytochrome b_{558}, and lane 2, solubilized sperm protein ($\approx 5x10^6$ cells). The arrow indicates the expected position of the α subunit of cytochrome b_{558}. Panel B shows a blot probed with an antibody against the 91kDa, ß subunit of cytochrome b_{558}. Lanes 1 and 2 as before. Again, the arrow shows the expected position of the ß subunit of cytochrome b_{558}. Both antibodies failed to cross-react with the sperm protein.

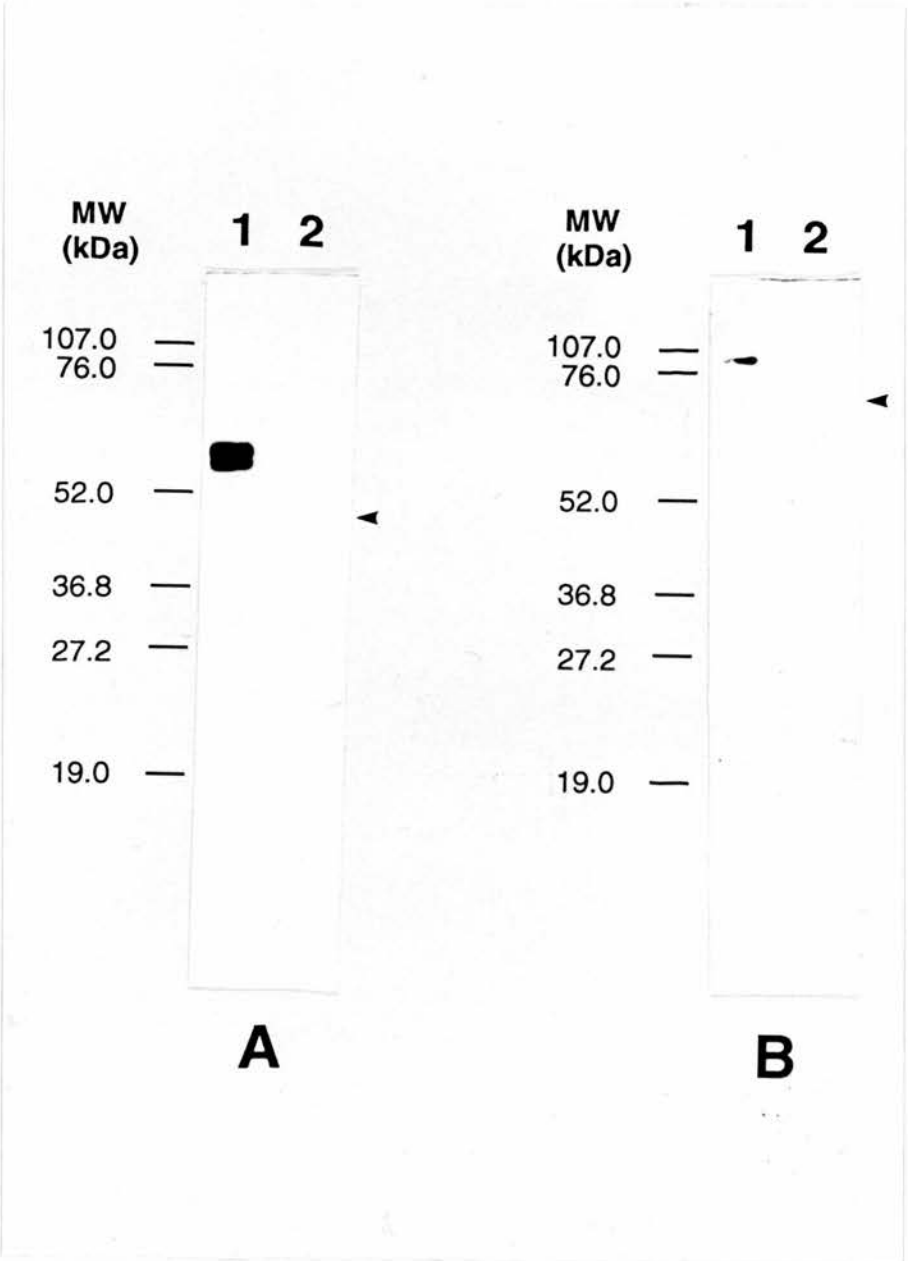

Figure 5.13 Western blots of SDS solubilized, human sperm protein, electrophoresed under reducing conditions. Panel A shows a blot probed with an antibody against the p47^{phox} component of the leucocyte NADPH oxidase. Lane 1, purified p47^{phox}, and lane 2, solubilized sperm protein ($\approx 5x10^6$ cells). The arrow indicates the expected position of p47^{phox}. Panel B shows a blot probed with an antibody against the p67^{phox} component of the leucocyte NADPH oxidase. Lane 1 purified p67^{phox}, and lane 2 as before. Again, the arrow shows the expected position of p67^{phox}. Both antibodies failed to cross-react with the sperm protein.

5.4 Discussion

Activation of the phagocytic leucocyte NADPH oxidase complex involves the phosphorylation of a key oxidase component, p47-^{phox}, on its' serine or threonine residues. One of the ways this may be brought about is by the activation of protein kinase C (Wymann *et al*, 1987), although other second messenger pathways are also thought to bring about p47-phox phosphorylation, and hence, oxidase activation (Morel *et al*, 1991- review).

In vivo, PKC is activated by diacylglycerol (DAG) and fatty acids, the activation of PKC stemming from the ability of these cellular second messengers to increase the affinity of PKC for calcium; calcium binding by PKC being required for its kinase activity. *In vivo*, DAG is formed by the phospholipase C (PLC) -dependent cleavage of phosphatidylinositol 4, 5, bisphosphate (PIP_2), which occurs upon agonist stimulation. Inositol 1, 4, 5-triphosphate ($InsP_3$) and DAG are then generated and can both go on to activate PKC. $InsP_3$, which is released into the cytosol, can activate PKC by its' ability to increase intracellular calcium concentrations, through the release of calcium from calcium storage organelles. DAG, which remains in the cytoplasmic half of the plasma membrane, activates PKC by increasing its' affinity for calcium and inducing its' translocation from the cytosol to the plasma membrane (Alberts *et al*, 1989). Once residing in the plasma membrane in an active state, PKC is able to phosphorylate the p47^{phox} component of the NADPH oxidase, and thus facilitate its' translocation to the plasma membrane, where it becomes assembled along with the other components of the NADPH oxidase complex, to form the active NADPH oxidase, which is then competent to generate superoxide (Morel *et al*, 1991). A schematic diagram showing the molecules, and some of the basic signal transduction mechanisms, involved in NADPH oxidase activation is shown in Figure 5.14.

In vitro, it has been well established that the phorbol ester, phorbol 12-myristate-13-acetate (PMA), is able to activate the NADPH oxidase of phagocytic leucocytes and thus, elicit a vigorous respiratory burst, along with concomitant ROS generation. This was first shown by Repine *et al* (1974) and has since been confirmed by numerous other researchers (e.g. Wymann *et al*, 1987; Rossi, 1986; Dieter, 1992; Curnutte *et al*, 1994).

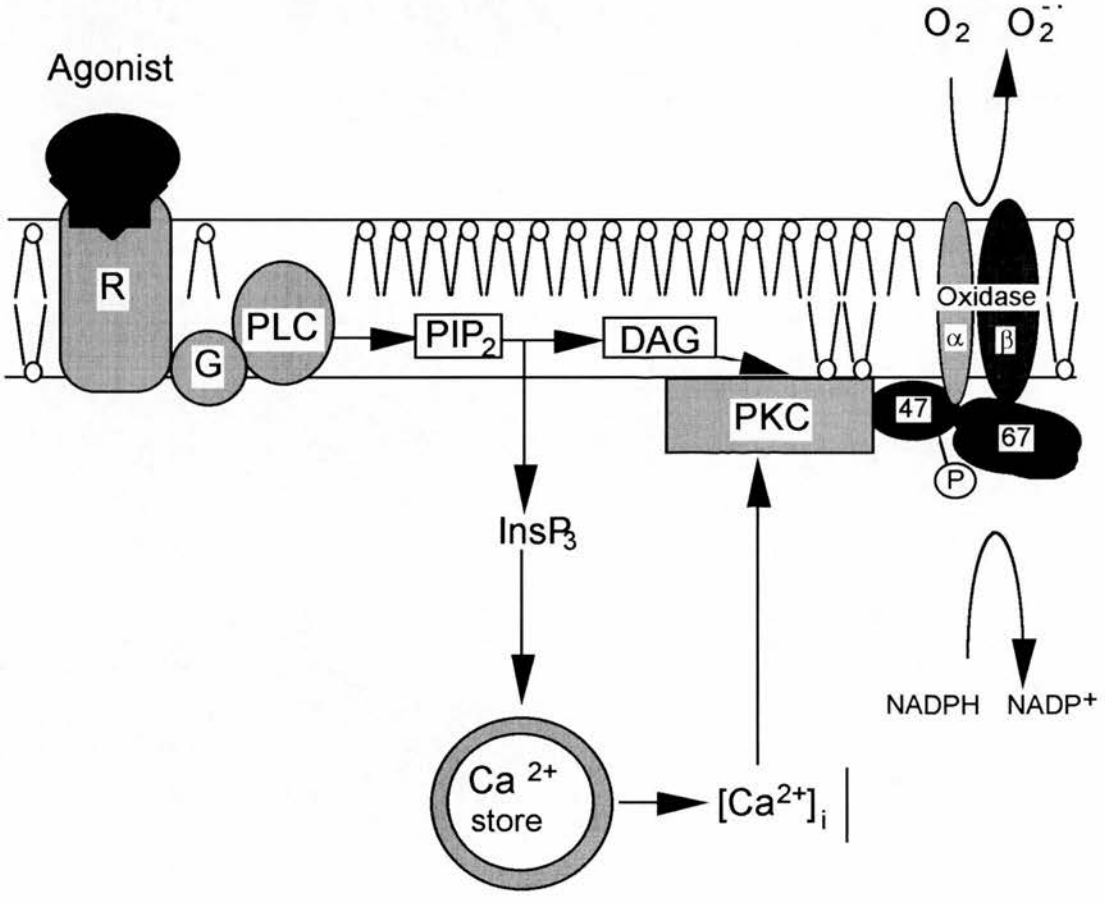

Figure 5.14 A very basic schematic diagram of some of the cellular signalling processes, and molecules involved, in the generation of the superoxide anion by the NADPH oxidase complex of phagocytic leucocytes. For further details see text.

PMA is a tumour promoting phorbol ester that has been shown to traverse the plasma membrane and mimic the action of DAG on PKC, thus directly activating it (Castagna *et al*, 1982). It has therefore, been assumed that PMA stimulates ROS generation by its' action on PKC. This theory has been substantiated by numerous studies showing that the PMA response can be

inhibited by specific inhibitors of PKC, such as staurosporine (Tamaoki *et al*, 1986; Koenderman *et al*, 1989), H7 (1-[5-isoquinolinesulfonyl]-2-methylpiperazine) (Fujita *et al*, 1986; Curnutte *et al*, 1994) and 1-0-hexadecyl-2-0-methylglycerol (Kramer *et al*,1989).

It has also been shown that PMA stimulates ROS generation by human spermatozoa (Aitken *et al*, 1992a and b; Aitken and Buckingham, 1992; Krausz *et al*, 1992; Aitken *et al*, 1993a; Aitken *et al*, 1994b; Krausz *et al*, 1994). Aitken *et al* (1992b) speculate that the response to PMA is specifically due to its effect on PKC, as the non-PKC-stimulating phorbol ester, 4α-phorbol 12,13-didecanoate, did not stimulate ROS generation. However, it remains to be conclusively proven that the action of PMA on human spermatozoa, i.e. inducing ROS generation, is a result of PKC activation. In order to address this issue the abilities of two inhibitors of PKC, staurosporine and H7, to block PMA-induced ROS generation by human spermatozoa were investigated. Both of these compounds inhibited PMA-induced ROS generation by human spermatozoa. The first compound tested was staurosporine, which is a very specific inhibitor of PKC, and has been used extensively to study the involvement of PKC in numerous cellular events, including its' role in ROS generation by phagocytic leucocytes (Koenderman *et al*, 1989). Staurosporine inhibits PMA-induced ROS generation by leucocytes, and similarly inhibited PMA-induced ROS generation human spermatozoa. The other PKC inhibitor tested was the less specific H7, which has also been shown to inhibit PMA-induced ROS generation by leucocytes (Curnutte *et al*, 1994). Although H7 is not as specific in its' action as staurosporine, i.e. it inhibits PKA and PKG as well as PKC (Garland, 1987), but the data generated from the two compounds, viewed in tandem, does go a substantial way in proving that PMA-induced ROS generation by human spermatozoa is PKC-dependent. This observation lends further support to the hypothesis that human spermatozoa generate ROS by a mechanism which is similar in some respects, to that of the NADPH

oxidase complex of leucocytes. However, it does remain for the actual role of PKC, in stimulating ROS generation by human spermatozoa to be determined.

Is its' role to assemble and activate the oxidase in human spermatozoa, like in leucocytes, is it somehow increasing the affinity of the sperm oxidase for the available endogenous NADPH, or is it somehow increasing the supply NADPH by increasing the activity of the hexose monophosphate shunt? These suggestions may not be entirely exclusive of one another. With regard to the first suggestion, the PMA response of human spermatozoa may be an artefactual response, dependent upon the retention of excess, residual cytoplasm. Human spermatozoa do generate ROS without the apparent need for any prior activation or assembly, when supplied with an exogenous supply of NADPH, and PKC-dependent protein phosphorylation does not appear to be involved in this phenomenon. The NADPH response may be representative of the physiological situation, in which ROS generation is possibly controlled by the availability of substrate from external sources. It is reasonable to assume that in a cell usually so devoid of cytoplasm, and hence the enzymes responsible for NADPH generation, a system for the regulation of ROS generation outwith that of substrate availability, is probably quite unnecessary. It is possible that the PMA response of human spermatozoa is a response indicative of pathological or abnormal spermatozoa, in which excessive cytoplasm, and hence capacity to generate NADPH has been retained, and that the PMA somehow stimulates, non-physiological, generation of ROS using this endogenous substrate. The fact that a modulator of protein phosphorylation (PMA) had no effect on ROS generation stimulated by exogenous NADPH, but that other such modulators, i.e. okadaic acid and PKC inhibitors, did significantly affect ROS generation in response to PMA, presents the possibility that two separate mechanisms, or at least two separate regulatory mechanisms, exist, mediating ROS generation by human spermatozoa by two distinct pathways.

Although, these data raise the possibility that human spermatozoa possess more than one mechanism for ROS generation, i.e. one requiring protein phosphorylation for activity and one that is merely dependent on substrate availability, it may be that by adding excessive substrate, i.e. NADPH, which *in vivo* the cells may not encounter, any delicate control mechanisms, such as those based upon the level of protein phosphorylation, may be made redundant, due to a swamping of the system with excessive substrate. It is plausible that in an 'inactive' state the oxidase has a low affinity for NADPH which, when exogenous NADPH is supplied, becomes immaterial due to a sudden increase in the availability of NADPH to the binding site of the oxidase. Upon activation of PKC, e.g. by PMA, the oxidase may become phosphorylated which, in some way, leads to an increase in its affinity for NADPH, so that the very low concentrations of endogenous NADPH available to the oxidase, become sufficient to effect ROS generation. However, this is merely speculation, and it remains for experimental evidence to be provided before this issue can be addressed, and such experiments are beyond the scope of this thesis.

It is also possible that PKC activation and hence, protein phosphorylation, leads to an increase in the activity of the HMS. This theory does have some basis in fact. It has been shown in phagocytic leucocytes, that PMA induced oxidase activity is associated with an increase in the level of hexose monophosphate shunt activity and thus NADPH generation (Cross and Jones, 1991), which may be due to an increased level of $NADP^+$, observed upon activation of the oxidase. It is also widely appreciated that phosphorylation by PKC, can lead to enhancement of the activity of many different enzymes, one of the reasons for this being a lowering of the requirements for co-factors and substrates (Cohen, 1992); this could be true for the enzymes of the HMS as well as the oxidase itself.

In addition to the activating effect of PKC on ROS generation, protein phosphatases also appear to be operating, simultaneously down-regulating PKC-dependent ROS generation. This hypothesis is based on the ability of okadaic acid to enhance the PMA response of human spermatozoa. Okadaic acid is a tumour promoting polyether fatty acid (Suganuma *et al*, 1988) which was first isolated from the marine sponges *Halichondria okadaii* and *Halichondria melanodocia* (Tachibana *et al*, 1981). It is a potent and specific inhibitor of serine and threonine protein phosphatases, types PP1 and PP2A, with little or no activity against other protein phosphatases or protein kinases. PPI and PP2A have homologous catalytic subunits and are the main phosphatases responsible for reversing the action of PKC (Cohen and Cohen, 1989), but have greatly differing IC_{50} values for okadaic acid (the IC_{50} being the concentration of the inhibitor required to reduce the activity of the phosphatase to 50% of its' maximal level). The IC_{50} of PP1 is between 10 and 20nM, whereas the IC_{50} of PP2A is only a fraction of this, around 0.1nM. This difference in the IC_{50}'s allows the two phosphatases to be easily distinguished from one another. Hence, from the concentrations required to affect ROS generation by human spermatozoa, in response to PMA, i.e. 10nM, it can be concluded that it is PP1 that is involved in the down regulation of ROS generation by human spermatozoa, and it is likely that the phosphatase's normal role is to de-phosphorylated the component of the sperm oxidase which PKC phosphorylates. Studies using another protein phosphatase inhibitor, Calyculin A, have also shown that human spermatozoa contain type PP1 and PP2 protein phosphatases, and that they are involved in the capacitation process (Furuya *et al*, 1993b). The authors of this paper also suggest that it is PP1 that is the dominantly active protein phosphatase in human spermatozoa.

These results are consistent with some of those obtained for the NADPH oxidase of phagocytic leucocytes. Some studies have shown that okadaic acid blocks de-phosphorylation of $p47^{phox}$, and in doing so enhances ROS

generation by whole cells in response to PMA. Enhancement was also observed in a cell free system, in response to arachidonic acid, although this situation was only seen when sub-optimal levels of the stimuli were employed (Ding and Badwey, 1992). However, it should be pointed out that in other studies, okadaic acid was found to suppress PMA-induced ROS generation by phagocytic leucocytes, but enhance the response to FMLP (Garcia *et al*, 1992). The authors of neither of the studies suggest reasons for the discrepancies between the results, but the authors of the latter study do make the point that there are numerous signal transduction pathways involved in NADPH oxidase activation and regulation.

Thus, from the effects of these various modulators of protein phosphorylation on PMA-induced ROS generation by human spermatozoa, it can be concluded that protein phosphorylation is definitely involved in this PMA-induced phenomenon. However, the involvement of protein phosphorylation in ROS generation is not universal, i.e. not all cell types that generate ROS appear to do so by a mechanism that involves protein kinase C dependent protein phosphorylation. Fibroblasts, though generating ROS by a mechanism that shares many of the characteristics of the NADPH oxidase, e.g. a low potential cytochrome b_{558}, do not appear to utilize PKC in this phenomenon. PMA does not stimulate significant levels of ROS generation by these cells, nor does staurosporine inhibit ROS generation by fibroblasts (Meier *et al*, 1991; Emmerdorffer *et al*, 1993). Similarly, ROS generation by glomerular mesangial cells does not involve PKC, again PMA having no significant stimulatory effect on ROS generation (Radeke *et al*, 1991). An NADPH-dependent oxidase system that does have an absolute requirement of PKC is the sea urchin oxidase of fertilization, i.e. the oxidase responsible for establishing the fertilization envelope, which is a hard impenetrable membrane responsible for ensuring the block to polyspermy (Shapiro, 1991). Thus it may be that the 'oxidase' responsible for ROS generation by human spermatozoa

shares the properties of more than one cellular system capable of ROS generation. On the one hand, like the NADPH oxidase of phagocytic leucocytes its' activity can be modulated by compounds that alter levels of protein phosphorylation, but on the other, protein phosphorylation is not an absolute requirement for ROS generation by human spermatozoa, as is the situation in fibroblasts and glomerular mesangial cells. The non-PKC requiring ROS generating activity by human spermatozoa and other cells may be, in part, due to the ability of NADPH to somehow activate NADPH oxidase-like activity, itself. It has been reported that NADPH not only acts as a specific reductant for superoxide generation, but also acts as a cofactor, that conformationally modifies the oxidase system, resulting in the formation of a superoxide generating state of the oxidase (Fujii and Kakinuma, 1991).

Leaving aside the very complex area of regulation of human spermatozoa ROS generation, let us now consider the cellular components involved in this cellular process. The development of a very specific and potent inhibitor of the flavoprotein component of the NADPH oxidase, has allowed the evaluation of the presence, and role for a flavoprotein component in the ROS generating complexes of other cells. This compound is diphenylene iodonium (DPI), and its' structure is shown in Figure 5.15. This compound is a lipophilic reagent that is an efficient inhibitor of ROS production by the NADPH oxidase of phagocytic leucocytes (Cross and Jones 1986; Ellis *et al*, 1988), and it has recently been shown to inhibit the generation of nitric oxide, through its' action on the enzyme nitric oxide synthase (Wang *et al*,1993).

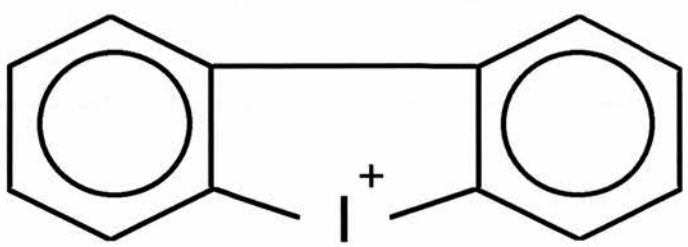

Figure 5.15 The structural formula of diphenylene iodonium.

Iodonium compounds are known to inhibit the activity of various flavoproteins and at least one haem-protein. DPI and other iodonium halides, are potent arylating agents, i.e. they mediate the addition of phenyl rings, and undergo thermal and photochemical cleavage under relatively mild conditions, and react readily with organo-metallic compounds, e.g. haem-proteins and flavoproteins. Arylation of haem-proteins and flavoproteins, probably occurs by a radical mechanism, whereby the inhibitor abstracts an electron from the reduced haem or flavoprotein, to form a radical which then adds back directly onto the prosthetic group or adjacent protein groups at or near the active site, to form adducts (O'Donnell *et al*, 1993). There is currently some debate as to which redox site of the NADPH oxidase is actually sensitive to DPI inhibition (O'Donnell *et al*, 1993; Doussière and Vignais, 1991; Doussière and Vignais, 1992), but it is probable that although both the haem of cytochrome b_{558} and the flavoprotein component of the oxidase are sensitive to DPI action, it is in fact the flavoprotein that is the actual site of inhibition (O'Donnell *et al*, 1993). Support for the flavoprotein being the site of DPI action is available from many studies showing that radio-labelled DPI covalently binds to a 45kDa flavoprotein component of the NADPH oxidase of phagocytic leucocytes (Cross and Jones, 1986), macrophages (Hancock and Jones, 1987) fibroblasts (Meier *et al*, 1991), and glomerular mesangial cells (Radeke *et al*, 1991). However, consensus has eventually been reached as to the mode of action of the DPI, with the above described mechanism being accepted, with the addition that the oxidase must be in an active, reduced form, i.e. electrons flowing through the system, for DPI-dependent inhibition to occur (O'Donnell *et al*, 1993; Doussière and Vignais, 1992).

Not only does DPI inhibit ROS generation by phagocytic leucocytes, it has also been shown to inhibit ROS generation by fibroblasts (Meier *et al*, 1991), by glomerular mesangial cells (Radeke *et al*, 1991), and by thyroid plasma membranes (Dème *et al*, 1994). Due to the widespread activity of DPI

on ROS generating systems, it seemed rational to determine whether this compound could inhibit ROS generation by human spermatozoa as well. The results obtained in this thesis have shown that DPI does inhibit ROS generation by human spermatozoa, and the concentrations required to inhibit ROS generation, in response to both PMA and exogenous NADPH, were both low enough, i.e. < 10μM, to confer a specific action of the DPI on the flavoprotein component of an NADPH oxidase-like system. Exceptionally low concentrations of DPI were capable of almost completely inhibiting ROS generation by human spermatozoa in response to PMA, i.e. 1μM reducing the level of PMA-induced ROS generation to approximately 1% of the non-DPI control. When spermatozoa were stimulated to generate ROS with exogenous NADPH the levels of inhibition observed were not as great as those achieved in the PMA system, i.e. 1μM DPI inhibiting NADPH-induced ROS generation by only 40%, and even 10μM only inhibiting by 65%. However, this was to be expected as DPI is thought to be a competitive inhibitor, with respect to NADPH (Yea *et al*, 1990), although it has more recently been reported, in two separate studies, that DPI is not competitive with respect to NADPH (O'Donnell *et al*, 1993; Doussière and Vignais, 1992).

In support of DPI being competitive with respect to NADPH, although it has not been conclusively shown that DPI binds to the NADPH-binding site of the NADPH oxidase, radioactively-labelled analogues of NADPH do label a 45kDa protein in phagocytic leucocytes, which is the same size as the protein which binds radio-labelled DPI (Takasugi *et al*, 1989; Cross and Jones, 1986). The work presented in this thesis supports the hypothesis that DPI is a competitive inhibitor, and would seem to contradict the more recent findings.

Even though DPI, a potent and specific inhibitor of the NADPH oxidase, inhibits ROS by human spermatozoa, in a seemingly specific fashion, can we interpret this as evidence for the existence of a similar oxidase in these cells? Firstly, DPI at higher concentrations is known to inhibit other enzymes, e.g.

mitochondrial NADH-dependent dehydrogenase (Ragan and Bloxham, 1977), so it's specificity for the NADPH oxidase is not absolute. Moreover, it is possible that the action of DPI results from an interaction with a FAD containing component, i.e. a flavoprotein, present in the sperm oxidase, but that none of the other components of the leucocyte NADPH oxidase are present in human spermatozoa, as is the situation in the hydrogen peroxide generating, NADPH oxidase of the thyroid plasma membrane (Dème *et al*, 1994).

Dealing firstly with the involvement of a FAD containing protein in human spermatozoa ROS generation, quinacrine, a FAD antagonist, which has a very similar structure to FAD, has been shown to inhibit ROS generation by phagocytic leucocytes (Bellavite *et al*, 1983; Tauber and Simmons, 1985; Cross *et al*, 1984). This compound was also used in assays of ROS generation by human spermatozoa, and addition of this compound to the spermatozoa resulted in inhibition of ROS generation, in response to both PMA and NADPH. This may imply, that like the NADPH oxidase of phagocytic leucocytes, a flavoprotein is a component of the ROS generating mechanism of human spermatozoa and this would support the DPI data. However, we must treat these quinacrine data with some caution, as quinacrine is not solely an antagonist of FAD. Quinacrine has many other inhibitory properties, e.g. inhibiting phospholipase A2; inhibiting cyclooxygenase; inhibiting various protein kinases including PKC and PKM; and it also inhibits the metabolic processes involved in NADPH generation, i.e. the hexose monophosphate shunt (Cross, 1990). Thus, quinacrine could possibly be acting at many points in a system responsible for ROS generation, and it cannot be assumed to be having a single, specific affect. However, when viewed in tandem with the DPI results, it is reasonable to hypothesise that a FAD-containing protein is, very probably, involved in ROS generation by human spermatozoa.

Thus, ROS generation by human spermatozoa is regulated, in some instances, by protein phosphorylation, and is susceptible to inhibition by inhibitors of flavoproteins. Since this suggests some similarities with the NADPH oxidase of phagocytic leucocytes, and the next logical step would be to determine whether homologous components of the NADPH oxidase of leucocytes are actually present in human spermatozoa. This investigation was conducted on two different experimental fronts: one based upon the unusual spectral characteristics of the cytochrome b_{558}, and the second involving a more molecular approach, i.e. detection of the specific proteins of the NADPH oxidase, employing the technique of Western blotting.

Let us deal firstly with the spectral analyses of human sperm membranes. This form of analyses failed to detect a low potential cytochrome such as b_{558} in human sperm plasma membrane preparations or in a crude/total membrane preparation of human spermatozoa. The quantities of the membranes used were comparable to those used in experiments on other cell types, in which the cytochrome b_{558} was detected (Cross *et al*, 1981; Meier *et al*, 1991; Radeke *et al*, 1991). It may be the case that the cytochrome is expressed at such low levels in human spermatozoa that much greater concentrations of membrane protein would be required for the spectral detection of the cytochrome b_{558}. Another possibility is that as the membranes were prepared in Edinburgh and then transported to Bristol before the analyses were performed, the samples may have become degraded in transit. This is very possible, as even in the crude membrane preparations only very low quantities of mitochondrial cytochromes were detected. Looking closely at the traces obtained with the crude membrane preparations (Figure 5.11) and comparing then with the traces in Figure 5.1, it can be seen that there do appear to be peaks at around 428 and 418nm, which are indicative of cytochrome b_{558}, but as there are no peaks (reduced minus oxidised) or troughs (reduced minus reduced + CO) on the traces at 558nm, this cannot really be taken to indicate

the presence of cytochrome b_{558}. Thus, these results have failed to demonstrate that a cytochrome b_{558} is present in human spermatozoa, but due to various factors surrounding these experiments, further studies are probably required to fully establish this point. At this juncture, the results cannot be taken to conclusively prove that a low potential cytochrome, similar or the same as that present in the NADPH oxidase of phagocytic leucocytes, is not present in human spermatozoa.

To address this point further a much more sensitive technique was employed, i.e. Western blotting of human sperm proteins, and probing with polyclonal antibodies against the NADPH oxidase of human phagocytic leucocytes. However, again the cytochrome b_{558} was not detected, neither the α nor the ß sub-unit, and nor were any of the other components of the leucocyte NADPH oxidase, i.e. $p47^{phox}$ or $p67^{phox}$. Three of the antibodies used gave positive results with the control protein samples, i.e. purified NADPH oxidase components, although the bands detected with the antibodies did not appear to be the correct molecular weights, though this may have been due to non-ideal electrophorectic conditions, due to some modification of the oxidase proteins during transport to our lab from London, or during their storage here prior to electrophoresis. The antibody against the ß sub-unit of cytochrome, completely failed to cross-react with the positive control. As the antibodies used in these experiments were polyclonal in nature, i.e. consisting of numerous different clones recognising many different antigens present on the denatured oxidase proteins, one would have expected that even if some differences existed between the molecules of the two different cell types, any reasonably similar molecules present in the spermatozoa would have been detected. However, due to the antibodies not reacting in the expected fashion, even with the positive control protein preparations, the results cannot be taken to conclusively prove that human spermatozoa do not contain any of the components of the NADPH oxidase of phagocytic leucocytes, and further

studies will have to be undertaken with antibodies against the leucocyte NADPH oxidase, of a more reliable nature, to show one way or another if human spermatozoa contain immunologically similar components.

Quite recently it has been shown that the NADPH oxidase of human fibroblasts does not actually contain components identical to those of the leucocyte oxidase (Meier *et al*, 1993). Even though these cells were shown to apparently contain cytochrome b_{558} by spectral and immunological analyses using polyclonal antibodies, it has now been shown that the cytochrome is not the cytochrome b_{558} present in the NADPH oxidase of phagocytic leucocytes. In the studies conducted by Meier *et al* (1993), numerous monoclonal antibodies raised against various epitopes of the cytochrome b_{558} were tested for their ability to cross react with fibroblast proteins. None of them did, showing that the cytochrome b_{558} of fibroblasts and that of leucocytes were actually, immunologically distinct. The study also reported on the cytochrome b_{558} content of the fibroblasts of a patient suffering from X-linked chronic granulomatous disease (CGD). CDG is a condition that is characterized by the absence of NADPH oxidase activity in the phagocytes of people suffering from it (Tauber *et al*, 1983). The X-linked form of the disease has been shown to be due to mutations in the gene encoding the ß subunit of the cytochrome b_{558} (Royer-Pokora *et al*, 1986; Teahan *et al*, 1987). Meier and her colleagues showed that the fibroblasts of a patient having X-linked CGD, had a normal content of cytochrome b_{558}, and were able to generate similar levels of superoxide as the fibroblasts of normal, healthy volunteers. In contrast, the phagocytes of this patient had only 10% of the normal levels of cytochrome b_{558}, and were able to generate less than 10% of the superoxide that normal phagocytes could. The authors postulate that if the two cytochrome b_{558} species were identical they would be under the same genetic regulation, and hence a mutation affecting one should also affect the other. The authors conclude, that the two cytochromes must be structurally (from the

immunological studies) and genetically distinct from one another. The fact that the cytochromes in fibroblasts and leucocytes are different proteins with different genes is in accordance with the different regulation of the two NADPH oxidase systems. Some potent stimulants for the leucocyte NADPH oxidase cause no, or only minor stimulation in fibroblasts and vice versa. In this instance, the use of different systems in leucocytes and fibroblasts is physiologically useful because of the different roles the two systems play. Whereas the NADPH oxidase system in leucocytes has an essential role in defence against infectious agents, it has regulatory functions in fibroblasts.

The same could possibly apply to the NADPH oxidase system of human spermatozoa, even though some similar control mechanisms do appear to be involved in the systems, e.g. PKC. If ROS generation has a true regulatory function in sperm physiology then, obviously, the normally low levels of ROS generated by these cells are not going to be damaging to the cells, or compromise their functional integrity. Thus, in this instance, rigorous control mechanisms regulating the assembly and activation of the oxidase are unnecessary, as even at maximal stimulation it is unlikely that the 'normal' spermatozoon would be capable of generating levels of ROS toxic to itself or to the surrounding cells and tissues. In fact it has recently been shown that even the relatively high levels of ROS generated by defective spermatozoa, do not actually damage 'normal' sperm cells present in a suspension (Plante *et al*, 1994). From the studies described in this thesis it does appear that complicated control mechanisms for ROS generation are not as necessary in human spermatozoa, as in leucocytes, and that the main control mechanism in operation is probably at the level of substrate, i.e. NADPH, availability. In fact studies have been carried out that have shown that the ability of human spermatozoa to generate ROS is indeed correlated to the cell's capacity for substrate, i.e. NADPH, generation (Aitken *et al*, 1994b). Thus, ROS generation by human spermatozoa can be described as being controlled, at least in part,

by substrate availability, and it can be appreciated that more complicated control mechanisms are normally unnecessary, and would possibly be metabolically costly to the cell. Of course, to fully address this issue the *in vivo* stimulator of ROS generation by human spermatozoa would have to be determined, and the pathways by which it acts deduced. Work in this field is now beginning to show some progress, with it recently being shown that an unknown, low molecular weight component of foetal cord serum (FCS) was able to stimulate superoxide generation by human spermatozoa (de Lamirande and Gagnon, 1995) and the same type of FSC preparation also stimulated sperm capacitation (de Lamirande and Gagnon, 1993a).

5.6 Summary and Conclusions

The results described in this chapter have shown that although the activity of the NADPH oxidase of human spermatozoa can be regulated by a mechanism similar to one present in phagocytic leucocytes, i.e. by PKC-dependent protein phosphorylation, it is not apparent if this mechanism has the same action in the two different cell types. In leucocytes PKC appears to regulate NADPH oxidase assembly and activation, (Wyman *et al*, 1987) whilst the sperm NADPH oxidase appears to require no prior assembly or activation for its activity, when supplied with exogenous NADPH, though in other circumstances, i.e. when utilizing endogenous NADPH, PKC-dependent protein phosphorylation does appear to have a regulatory role.

It does appear that, like phagocytes, sperm ROS generation involves an NADPH oxidase system with a flavoprotein component, but it has not been possible to demonstrate the existence of a low potential, b-type cytochrome in this system. Western blot analyses of spermatozoa have failed to reveal the presence of proteins immunologically similar to components of the NADPH oxidase of phagocytic leucocytes, and so it remains for any component of the NADPH oxidase of human spermatozoa to be identified. It is clear from these

studies that the NADPH oxidase of phagocytic leucocytes cannot be used as an accurate model for the ROS generating system of human spermatozoa, though it still may prove to be quite similar. Future work aimed at identifying the NADPH oxidase-like system in human spermatozoa will have to be based upon the characteristics of ROS generation by these cells, and one of the most useful of these would appear to be ability of human spermatozoa to generate the superoxide anion employing exogenous NADPH. The next section of this thesis will describe work carried out attempting to characterise, isolate, and identify the component or components of human spermatozoa that are responsible for NADPH-induced superoxide anion generation.

Chapter 6

Extraction, isolation and purification of ROS generating activity from human spermatozoa

6.1 Introduction

Human spermatozoa contain an enzymatic system capable of ROS generation. This system shares some of the functional characteristics of the NADPH oxidase of phagocytic leucocytes, i.e. utilizing NADPH as the electron donor to effect the univalent reduction of molecular oxygen to the superoxide anion; regulation of ROS generation by substrate availability and PKC-dependent protein phosphorylation; and the involvement of a flavoprotein, as indicated by inhibitor-based studies. However, human spermatozoa do not appear to contain components that are structurally similar to those of the leucocyte NADPH oxidase. Spectral analyses of the cytochrome content of human spermatozoa failed to reveal the presence of a low potential cytochrome b_{558}, which is a characteristic component of the NADPH oxidase of phagocytic leucocytes. Similarly, Western blot analyses of human sperm proteins with polyclonal antibodies raised against key components of the leucocyte NADPH oxidase, i.e. against the α and ß subunits of the cytochrome b_{558} and against the two cytosolic components, $p47^{phox}$ and $p67^{phox}$, failed to detect the presence of these components. Thus, it appears that human spermatozoa possess a cellular system capable of ROS generation that is at least structurally distinct from the NADPH oxidase of phagocytic leucocytes, and it remains for any of the components of this system to be identified.

Since the free radical generating systems in leucocytes and human spermatozoa are structurally dissimilar, a novel approach was adopted to characterize the biochemical basis of the sperm 'NADPH oxidase' activity. To

this end, the ability of human spermatozoa to generate the superoxide anion, when supplied with an exogenous source of NADPH, was exploited, along with the ability of non-denaturing detergents to solubilize cellular proteins without destroying their native structure and hence abolishing the enzymatic activity they may possess.

6.1.1 Detergents

Detergents are amphipathic molecules, i.e. they have hydrophilic (polar) and hydrophobic (non-polar) ends, which cause them to form micelles in water (Alberts *et al*, 1989). When mixed with intact cells, the detergent disrupts the lipid bilayer of the cell plasma membrane, bringing proteins present in the membrane into solution as protein-lipid-detergent micelle complexes. The hydrophobic region of the detergent binds to the hydrophobic region of the protein, thus displacing the membrane lipids and the hydrophilic region of the detergent brings the protein into solution. The membrane lipids also form micelles with the detergent, again resulting in their solubilization (Neugebauer, 1990a and b). Some strong ionic detergents such as sodium dodecyl sulphate (SDS), denature proteins by binding to their internal hydrophobic cores, rendering them inactive and unusable for functional studies. However, less hydrophobic proteins, requiring less stringent detergent treatment, can be solubilized in an active, though not necessarily entirely normal form, with much weaker non-denaturing detergents (Hjelmeland, 1990). Such detergents are commonly used to solubilize enzymes whilst maintaining their biological activity, and in fact, non-denaturing detergents have been used to prepare cell free, solubilized, NADPH oxidase preparations (Gabig *et al*, 1978, Parkos *et al*, 1987). Detergents that have been used to solubilize active, NADPH oxidase from phagocytic leucocytes were employed in the studies described herein, along with detergents known to be efficient at membrane protein solubilization, in retaining enzymatic activity

and at solubilizing cytochrome and flavoprotein containing proteins (Baines and Poole, 1985). It was hoped that through the use of these reagents, the solubilization of the ROS generating system from human spermatozoa might be achieved.

6.1.2 Superoxide-dependent NBT reduction

The ability of detergent-solubilized sperm protein extracts to generate the superoxide anion, when supplied with NADPH, was determined by the abilities of the various protein-extracts to support NADPH-induced, superoxide-dependent, reduction of nitroblue tetrazolium (NBT). Tetrazolium dyes, particularly NBT, have been used extensively as histological staining reagents for various dehydrogenase enzymes (Farber *et al*, 1956) and these early studies established that the staining reaction was linked to diaphorase-like abilities. More recently NBT has been found to react with the superoxide anion and therefore, has been used extensively as a probe for the generation of this oxygen radical in many *in vitro* systems (Thayer, 1990; Key *et al*, 1990; Hirai *et al*, 1992). The specificity of superoxide-dependent NBT reduction has been confirmed through inhibition studies employing the superoxide scavenger, superoxide dismutase (Oberly and Spitz, 1984; Key *et al*, 1990)) and by studies indicating that dicoumarol, a diaphorase inhibitor, does not inhibit the superoxide-dependent reduction of NBT (Thayer, 1990).

NBT is useful as a staining reagent for superoxide generation as in the presence of this molecule, NBT is reduced to an insoluble, blue/black, formazan precipitate (Tauber, 1981), as indicated by equation 1.

(1) $NBT^{++}, 2Cl^{-} + 4O_2^{\cdot -} + 4H^{+} \longrightarrow 2HCl + \underset{\text{blue/black (insoluble)}}{\text{Diformazan}} + 4O_2$ ($NBT^{++}, 2Cl^{-}$: yellow (soluble))

This superoxide-dependent NBT staining method has been used extensively to demonstrate the presence of NADPH oxidase activity in phagocytic leucocytes

(Tauber, 1981; Hirai *et al*, 1992) and other cell types, e.g. osteoclasts (Key *et al*, 1990), and cardiac myocytes (Thayer, 1990). Although all of these studies utilized either tissue sections or intact cells to demonstrate NBT-dependent staining of superoxide generating activity, NBT-reduction has also been used to visualize the activity of other enzymes and enzyme systems in solubilized protein preparations (Burgos *et al*, 1979; Mittler and Zilinkas, 1993; Skidmore and Beebee, 1991; Wang *et al*, 1990).

Once an optimal strategy for solubilization of the ROS generating activity of human spermatozoa, has been devised, the solubilized, components can be electrophoretically resolved, in an attempt to identify the individual proteins or protein-complexes responsible for the spermatozoon's capacity for ROS generation. This was accomplished by employing protein separation techniques that allow the resolution of complex protein mixtures whilst retaining the biological activity of the separated proteins or protein-complexes. Obviously, such separation techniques must be very gentle, and not contain elements that would lead to the denaturation of proteins or the dissociation of essential protein complexes. Two such techniques are isoelectric focusing (IEF), and non-denaturing or native polyacrylamide electrophoresis (PAGE) (Hanes and Rickwood, 1981).

6.1.3 Isoelectric focusing

Isoelectric focusing (IEF) is an electrophoretic technique for the resolution of molecules that differ in their net charge, i.e. according to their isoelectric point (Righetti, 1983). The proteins are allowed to migrate in a pH gradient and are separated via differences in their isoelectric points (pI's), i.e. at the position in the pH gradient at which the molecule has no net charge; at this pH, the protein molecules have no electrophoretic mobility and hence remain stationary. IEF is a highly sensitive method that not only identifies an intrinsic property of a protein, i.e. its pI, but which also has a very high resolving

power. The bands formed are narrow and sharply defined and proteins with similar or very close isoelectric points can be separated. Another advantageous feature of IEF is that diffusion of the separated proteins is not a problem. If a protein molecule diffuses away from its' isoelectric point it will immediately become charged and move back towards its isoelectric pH and remain focused there. In most other separation methods there is no force to counteract diffusion. The high resolving power of IEF, along with the fact that the separated protein molecules retain their biological activity, has lead to this technique being extensively used for the separation of the different isoforms of enzymes, e.g. lactate dehydrogenase (Pawlowski and Brinkman, 1992), and in their purification.

6.1.4 Non-denaturing PAGE

Non-denaturing PAGE, based upon the Ornstein-Davis PAGE system for native proteins (Ornstein, 1964; Davis, 1964) provides very high resolution and is a much more selective protein separation technique than IEF, as it fractionates molecules on the basis of their net charge, molecular weight and conformational shape (Jovin, 1973). As with IEF, non-denaturing PAGE allows the resolution of complex protein mixtures into discrete bands whilst maintaining the biological activity of the protein or protein complex. To utilize this technique, the isoelectric points of the proteins to be separated, must first be determined so that the buffers used in the system cover the correct pH range. The buffers used must result in the proteins having an overall negative charge so that during electrophoresis, the proteins of interest will firstly concentrate into a thin band in the stacking gel, and then subsequently, enter the resolving gel and be separated on the basis of their charge/mass ratio. This method has also been used to separate and analyse the isoforms of lactate dehydrogenase (Skidmore and Beebee, 1991; Salehi-Ashtiani and Goldberg, 1993), and to analyse diaphorase isozyme patterns in human spermatozoa

(Gavella and Lipovac, 1992).

Subsequent to the resolution of the solubilized, crude, protein mixtures, into individual components, the IEF or non-denaturing PAGE gels were subjected to the NBT staining protocol, in order to detect any bands on the gel capable of superoxide anion generation. Some gels, for comparisons, were also subjected to staining protocols for the detection of lactate dehydrogenase and diaphorase activities. Bands capable of superoxide anion generation were subsequently excised from the gel, the protein eluted, and then subjected to SDS-PAGE, to enable the determination of the molecular weight(s) of the protein(s) responsible for NADPH-induced superoxide anion generation by human spermatozoa.

An alternative method for the isolation of the proteins or protein complexes responsible for ROS generation by human spermatozoa was also used, based upon a chromatographic technique commonly employed in protein purification strategies. Chromatography is a technique that has many different variants, each one specialized to achieve separation on the basis of different, intrinsic properties that the proteins possess. The original chromatographic techniques were developed to separate small molecules such as sugars and amino acids but these techniques now have much wider application, and their development has revolutionized the biochemical analyses of complex mixtures of molecules (Alberts *et al*, 1989).

Proteins are most often separated by column chromatography, in which a mixture of proteins is passed through a column packed with a porous, solid matrix. The proteins in the heterogeneous mixture are retarded to different extents by their interaction with the matrix and thus they can be differentially collected, in their native functional states, as they flow out of the bottom of the column. According to the choice of matrix employed, proteins can be separated according to the following properties:-

1) Charge - ion-exchange chromatography
2) Hydrophobicity - hydrophobic chromatography
3) Molecular weight - size exclusion chromatography
4) Biological activity - affinity chromatography

Matrices that allow the separation of proteins according to the above properties are commercially available. Ion-exchange chromatography columns are packed with small beads that carry a negative or positive charge (cation and anion-exchangers respectively) and the proteins are fractionated according to the arrangement of charges on the surface of these molecules (Rossomando, 1990). Hydrophobicity chromatography columns are packed with beads that have protruding hydrophobic side chains, and separate proteins on the basis of their hydrophobicity, i.e. retarding the mobility of proteins with exposed hydrophobic regions that interact with the hydrophobic side chains of the chromatography matrix (Kennedy, 1990). Size exclusion chromatography columns are packed with tiny porous beads and cause the separation of protein molecules according to their size. Small molecules are able to enter the pores of the beads and thus, their flow through the column is retarded, whereas larger molecules cannot enter the beads and so flow through the column more rapidly, emerging from the column first. Matrices can be chosen to optimize the resolution of proteins of a particular molecular weight range (Alberts *et al*, 1989).

6.1.5 Affinity chromatography

All the chromatographic strategies described above are useful in separating and purifying proteins from protein mixtures that are partially fractionated already, but not those from very complex mixtures of proteins, such as exists in a whole sperm protein extract. It can be appreciated in these circumstances that more than one protein of a particular charge, hydrophobicity, and size will

very probably exist in such a mixture and hence, any given fraction will not contain a very homogeneous protein population. A chromatographic technique that can be used in such circumstances, is affinity chromatography (Wilchek *et al*, 1984). This chromatographic technique occupies a unique place in separation technology as it is the only method that can enable purification of almost any biomolecule on the basis of its biological function or individual chemical structure, and it does so in a relatively rapid fashion. Affinity chromatography is a technique in which the molecule to be purified is specifically and reversibly bound to a complementary ligand that has been immobilized on an insoluble support matrix. Unbound molecules will be washed away when a buffer, which does not interfere with the binding of protein of interest to the matrix ligand, is applied to the column. Bound molecules can then be eluted from the column in a specific or non-specific manner. Non-specific elution involves changing the composition of the column buffer so that the bound molecule becomes dissociated, e.g. by changing the pH, ionic strength or molarity of the buffer. Specific or affinity elution involves addition of a compound to the column buffer for which the molecule to be purified has a higher affinity than the ligand to which it is bound on the column matrix, e.g. an enzyme may be bound to an antagonist ligand and addition of the native substrate of the enzyme to the column results in its dissociation from the matrix and hence, its specific elution from the column. Matrix/ligand bound molecules with different native substrates will not be dissociated and eluted. Affinity chromatography is a very powerful technique and purification via this method often results in increasing the proportion of the protein of interest by several thousand fold. The recoveries are often very high and the functional, biological integrity of the molecule is generally not compromised (Wilchek *et al*, 1984). Spectacular separations have been achieved in which only one affinity chromatography step was required to enable complete purification of the molecule of interest. Affinity

chromatography has been given extreme flexibility through the development of a relatively simple technique for covalently attaching ligands of various types to polysaccharide matrices, i.e. via cyanogen bromide activation (Axen *et al*, 1967), and as a consequence of its exemplary specificity and time-efficient nature, affinity chromatography is often the first purification method of choice, provided a suitable ligand is available.

Affinity chromatography has been used to separate protein components involved in ROS generation, e.g. purification of the NADPH-binding domain of the NADPH oxidase (Umei *et al*, 1991; Yea *et al*, 1990) and in the purification of other NADPH-dependent enzymatic systems, e.g. nitric oxide synthase (Hope *et al*, 1991). Both these procedures involved the use of a structural analogue of NADPH, i.e. 2',5' adenosine diphosphate (2',5'ADP), covalently bound to a column matrix. Enzymes dependent on NADPH will bind to this compound but will be selectively displaced from it upon addition of NADPH to the system. Enzymes that have been purified to homogeneity utilizing affinity chromatography on 2',5'ADP-agarose include various NADPH-dependent dehydrogenases, transhydrogenases, reductases, oxidases and synthases (Wilchek *et al*, 1984). The affinity of the sperm superoxide generating system for NADPH was exploited in a similar fashion, in order to isolate and purify the superoxide generating system. The ability of this system to generate the superoxide anion when supplied with NADPH was also exploited to follow the purification of the active constituents. Subsequent to 2',5'ADP affinity chromatography, any fractions displaying superoxide generating activity, as indicated by NBT reduction, were subjected to SDS-PAGE in order to resolve the constituent proteins and then processed further, when indicated, by electro-elution.

6.2 Materials and methods

6.2.1 Cell preparation

Human semen samples were prepared as previously described and only the resulting sperm suspensions that were free of leucocyte contamination, used in the following experiments.

6.2.2 Solubilization and extraction of ROS generating ability from human spermatozoa

Pure human sperm suspensions were subjected to various protein extraction strategies. The protein extraction protocols employed involved various combinations of three extraction buffers and 5 non-denaturing detergents.

The buffers used were 1) the Tris-based extraction buffer used in previous experiments; 2) a flavin adenine nucleotide (FAD) containing buffer, based upon a buffer developed by Gabig *et al* (1978) used in the solubilization of NADPH oxidase activity (40mM Tris-HCl, pH 8.3; FAD, 0.4mM; $CaCl_2$, 3.2μM; NaN_3, 4.0mM); and 3) a HEPES based buffer (KCl, 100mM; NaCl, 10mM; HEPES, 10mM; EDTA, 1mM; dithiothreitol, 1mM; PMSF, 1mM; aprotinin, 30Ti U/ml), which is based again, upon one used in the extraction of components of the NADPH oxidase, in this instance, the cytochrome b_{558} (Parkos *et al*, 1987). The buffers were designated Tris buffer, FAD buffer, and MRB buffer respectively.

The detergents and concentrations of each used, were as follows: Triton X-100, 0.8%; CHAPS [3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate], 5mM; CHAPSO [3-(3-cholamidopropyl)dimethyl-ammonio-2-hydroxy-1-propanesulfonate], 5mM; n-octyl-ß-D-glucoside (OG), 1.1%; (all from Pierce, Rockford, IL, USA) and n-octyl-ß-D-thioglucoside (OTG), 1.1% (Calbiochem). The detergents were dissolved in the 3 different buffers, resulting in 15 different detergent solutions.

Initially, 20µl of each detergent solution was used to solubilize 20 x 10^6 spermatozoa. Briefly, 16 x 1ml aliquots of leucocyte-free, human sperm suspension were washed X3 in Tris washing buffer, as outlined in an earlier section of this thesis. The washed sperm pellets were then resuspended in 20µl of one of the detergent solutions or 20µl of PBS. The pellets in the detergent solutions were then treated as indicated in Figure 6.1.

To assess the efficacy of each of the detergent solutions in solubilizing active, NADPH-dependent superoxide generating ability, a dot blot of the solubilized human spermatozoa was constructed. Briefly, a 6 by 6 grid was made on nitrocellulose membrane (Hybond C-super, Amersham). Onto each square of the grid was applied 10µl of one of the solubilized sperm preparations, 10µl of the one of the detergent solutions, 10µl of the sperm-PBS suspension, 10µl PBS or 10µl BWW. The various samples were all applied 1µl at a time, allowing the nitrocellulose membrane to dry between applications. After application of the samples, and the complete drying of the nitrocellulose, the blot was processed to determine superoxide generating activity, employing the NADPH-induced, superoxide-dependent NBT staining technique described below, section 6.2.3.

6.2.3 Superoxide-dependent Nitroblue Tetrazolium (NBT) staining

Superoxide generating activity was determined by incubating the nitrocellulose blots or gels in an NBT staining solution. The staining solution was made up immediately prior to use and comprised of the following: Trizma 7.3, 50mM; NBT, 2.45mg/ml; NADPH, 1mM; dicoumarol (to block diaphorase activity), 100µM (10mM stock solution in 0.01N NaOH), (all from Sigma) in distilled H_2O. An appropriate volume of the stain was prepared to ensure complete coverage of the blot or gel, usually around 25mls, and stored protected from the light until use.

The blot or gel was immersed in the freshly prepared stain, and

incubated in it, protected from the light, at room temperature until a purple coloration/precipitation representing ROS generating activity became visible, usually after about 15 minutes. The activity on the blots was observed as a purple precipitate on a pinkish background, and on polyacrylamide gels as blue/black bands against a light purple, transparent background. Incubation in the staining solution for too long a period, resulted in a very dark background coloration that obscured the bands. Staining was terminated by rinsing the staining solution away with PBS, and then immersing the blots and gels in 5% acetic acid (BDH). In some instances, 1mg/ml SOD (Calbiochem) was added to staining solution, in order to demonstrate the superoxide-dependency of the NBT-staining.

The blots were subsequently allowed to dry and then photographed, whilst the gels were stored in the 5% acetic acid solution until a permanent photographic copy could be made.

6.2.4 Lactate dehydrogenase-dependent (LDH) NBT staining

Two protocols for LDH activity staining were employed; one based upon total LDH activity, and one based upon the activity of the testis-specific, LDH-C4 isoform of LDH.

Total LDH staining

Total lactate dehydrogenase activity was assessed, again using a NBT-staining method, according to the method of Pawlowski and Brinkman (1992) with a few modifications. In this instance the staining solution comprised of Trizma 7.3, 50mM; NBT, 2.45mg/ml; NAD^+, 1mM; phenazine methosulfate (PMS), 1mM; and lactic acid (sodium salt), 1mM (all from Sigma). The staining procedure was as for superoxide-dependent NBT staining except that staining was only allowed to proceed for between 5 and 10 minutes.

LDH-C4 staining

The protocol in this instance was almost identical to the total LDH activity staining except in place of lactic acid the LDH-C_4-specific substrate, hydroxycaproic acid was used (Burgos *et al*, 1979). In this instance the concentration of hydroxycaproic acid used was 5mM. Other than this modification the staining protocol was identical to that above.

6.2.5 Diaphorase-dependent MTT staining

The staining protocol involved in staining for diaphorase activity was similar to that for superoxide-dependent NBT staining except a completely different staining solution was used, this time based upon the ability of dehydrogenase enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble purple precipitate. The staining solution was made up as follows: Tris/HCl, pH 8.4, 250mM; 2,6, dichloroindophenol (DCIP), 0.001%; NADPH 0.05%; and MTT, 0.025% (Gavella and Lipovac, 1992).

6.2.6 Isoelectric focusing

Briefly, acrylamide gels were prepared containing Ampholine™ carrier ampholytes from Pharmacia, which are specially prepared mixtures of small amphoteric molecules that form a linear pH gradient when a charge is applied to the gel.

Isoelectric focusing was carried out using a LKB 2117 Multiphor II Electrophoresis System, according to the manufacturers instructions, and using isoelectric focusing accessories from LKB (LKB, Pharmacia). Isoelectric focusing of OTG solubilized human spermatozoa was carried out, briefly, as follows. 2mm polyacrylamide gels were prepared consisting of 5% bis-acrylamide (30% w/v, 19:1, Integra Biosciences, Northumbria Biologicals, Cramlington, UK), 3ml pre-blended Ampholine mixture, pH range 3.5-9.5, (Pharmacia), 0.35% (w/v) OTG (Calbiochem), and made up to 60ml with

distilled H_2O, and then polymerized with 300µl 10% (w/v) ammonium persulphate (BioRad), dissolved in distilled H_2O. The gels were cast and allowed to polymerize for at least 1 hour before focusing was begun. The anode solution used was 1M orthophosphoric acid, and the cathode solution was 1M sodium hydroxide: both were purchased from BDH. The gels were then pre-focused for one hour, at 25W, prior to sample application. The solubilized protein samples were applied using sample application pieces, 5 pieces being used, one on top of another, so to allow application of up to 100µl of sample. 5µl of BioRad IEF standards were also applied to the gel (BioRad), and these consisted of phycocyanin, pI 4.65; ß-lactoglobulin B, pI 5.10; bovine carbonic anhydrase, pI 6.00; human carbonic anhydrase, pI 6.5; equine myglobin, pI 7.00; lentil lectin (3 bands), pI's 8.20, 8.40, and 8.60; cytochrome c, pI 9.6. Isoelectric focusing was started and allowed to proceed at 25W for approximately 2 hours, or until the IEF standards appeared to have reached their stationary points. The IEF gels were then subjected to the NBT staining protocol described below, or to Coomassie blue staining (Chapter 3).

6.2.7 Non-denaturing PAGE

Protein extracts of human spermatozoa were also subjected to non-denaturing PAGE in order to separate the proteins, whilst maintaining their biological activity. From the IEF, the pI's of the proteins of interest were determined and suitable buffers for the non-denaturing PAGE, to enable optimum separation of the proteins, devised. The buffers used were, in fact, the same as those used in SDS-PAGE, since the proteins of interest possessed pI's that were compatible with the pH's of the SDS-PAGE buffers. For non-denaturing PAGE 5% polyacrylamide gels with 3.5% stacking gels, were prepared, as previously described for SDS-PAGE, except that SDS was omitted from the polymerization mixtures, and 0.35% (w/v) OTG included instead.

Bromophenol blue (BDH), at a concentration of 0.002% (w/v), was added

to the OTG solubilized samples, which were applied into the wells of the stacking gel, and then the electrophoresis was performed as described earlier, employing a current of 30mA through the stacking gel and 20mA through the resolving gel. The electrophoretic run was allowed to proceed until the bromophenol blue tracking dye almost reached the bottom of the gel.

Following electrophoresis, gels were either stained for ROS generating activity via the NBT staining protocol, or stained for overall protein via silver or Coomassie blue staining. Gels were stored in 5% acetic acid until permanent, photographic records could be made

6.2.8 2',5' ADP affinity chromatography of solubilized sperm proteins

Total human sperm protein was solubilized and extracted from human spermatozoa employing the OTG extraction protocol described above. For affinity chromatography 2µl of extraction buffer was used per 10^6 spermatozoa, and the extracts were used immediately or stored at -20°C until use. Frozen extracts were thawed on ice prior to the affinity chromatography, which was conducted according to the method of Umei *et al* (1991), with a few minor modifications.

The affinity matrix used was agarose beads, cross linked with 2',5' adenosine diphosphate (2', 5' ADP) (Sigma). An agarose slurry was prepared by mixing approximately 625mg of the cross-linked, 4% beaded agarose with 20ml of the column buffer (50mM HEPES (Gibco) and 25% v/v ethylene glycol (Sigma), adjusted to pH 8.0 with concentrated HCl). All buffers were de-gassed, under vacuum, prior to use. The agarose slurry was allowed to swell in the column buffer for 15 minutes, with gentle mixing, before being poured into the column. The chromatography column used was an Amicon G10 Moduline™ medium-pressure, borosilicate glass column (Amicon, Stonehouse, UK) which had an adjustable bed height of between 4-10mls. The swollen agarose was carefully poured into the column, using a glass Pasteur

pipette, gently releasing the slurry down the side of the column to prevent any air bubbles forming. Once all the slurry had been placed in the column, it was allowed to settle, in this case resulting in a column bed volume of 10mls. Once the agarose had settled the column was attached to a retort stand and connected to a LKB 2132 Microperpex® peristaltic pump, LKB UVICORD SII UV detector, and LKB 2212 HELIRAC fraction collector (all from LKB, Pharmacia). The column was washed through with a few column volumes of the HEPES-glycol column buffer at a flow rate of 10mls/hour, with the buffer maintained at around 4°C by incubating it in an ice bucket.

Immediately prior to the affinity chromatography run, the OTG protein extract was diluted 1:2 with HEPES-glycol buffer and then applied to the column at a rate of 10ml/hour. Generally, approximately 3ml of OTG sperm extract ($\approx$ 1500 x 10^6 spermatozoa, 2.25mg protein) was used per run, i.e. around 9mls of OTG extract/HEPES-glycol buffer mixture. At this point the fraction collector was started and 1ml fractions from the column collected. Once the entire sample had been applied to the column, HEPES-glycol buffer was run through the column at 10ml/hour until the all the unbound protein had been eluted, and the absorbance at 280nm had returned to the basal level. At this point, the affinity elution buffer was applied to the column. The elution buffer was the HEPES-glycol buffer with 5mM NADPH added. Due to the high cost of NADPH, only a 5ml pulse of this elution buffer was run through the column, and then the unadulterated HEPES-glycol buffer was again applied to the column. Throughout the column run, 1ml fractions were collected and stored at 4°C with the addition of 10µl of a protease inhibitor mixture (1mM AEBSF (4-(2-aminoethyl)-benzenesulphonylfluoride (Calbiochem), and 2mg/ml aprotinin (Sigma)), until assayed for superoxide generating ability (see below). After the absorbance at 280nm had returned to basal levels, i.e. after elution of bound protein, the column was regenerated using column buffer containing 1.5M NaCl (BDH), and then stored at 4°C in

buffer containing 0.002% of the anti-bactericidal agent, Thimerosal (Sigma). Prior to re-use the column was thoroughly washed through with HEPES-glycol buffer to remove the Thimerosal.

Determination of superoxide generating ability by column fractions

The affinity chromatography fractions were assayed for superoxide generating ability by an NBT-based spectrophotometric, microtitre plate method. The assay was based upon the same principles as the superoxide-dependent NBT staining protocol, and was carried out, briefly, as follows. 50µl of an NBT solution containing 2.45mg/ml NBT and 300µM dicoumarol in 50mM Trizma 7.3 (all Sigma) were placed in the required number of wells of a 96 well Nunclon™ microtitre plate (Nunc, Kanstrup, Denmark). 50µl of the affinity fractions to be assayed were placed into the wells along with the NBT reaction mixture, and then superoxide anion generation was stimulated by the addition of 50µl of a 2.5mg/ml NADPH solution, again in Trizma 7.3. Blank wells were prepared by using 50mM Trizma 7.3, column buffer, or affinity elution buffer, in place of the column fractions. The reactions were allowed to proceed in the dark, at 37°C for two hours, and then the absorbances of the wells, at 540nm, were measured using a Multiskan® MCC/340 spectrophotometric, microtitre plate reader (Labsystems, Basingstoke, Hants., UK). A standard curve was prepared using known amounts of the reduction product of NBT, i.e. nitroblue diformazan (NBD) and the concentration of NBD plotted against the absorbance at 540nm. The absorbances of the affinity chromatography fractions were compared with those of the standard curve, and the quantity of NBD formed determined. The actual quantity of superoxide formed was determined from the following equation:-

$$NBT^{++}, 2Cl^{-} + 4O_2^{-\cdot} + 4H^{+} \longrightarrow 2HCl + \underset{\textbf{(NBD)}}{\text{Diformazan}} + 4O_2$$

The above equation shows that 4 moles of the superoxide anion are required to

reduce 1 mole of NBT to NBD. Thus by calculating the amount of NBD formed in each of the wells of the microtitre plates we can determine the amount of superoxide anion generated by each fraction, according to the equation below:-

$$\text{moles NBD} \times 4 \times 20/2 = \text{moles superoxide. ml}^{-1}\text{. hour}^{-1}.$$

The chromatographic data has been presented as representative chromatography runs, showing the UV absorbance at 280nm, superoxide anion generation, and fraction number (Figure 6.6).

NADPH-eluted fractions with significant superoxide anion generating ability, were individually concentrated using Amicon Centricon microconcentrators as described in section 6.2.11, and subjected to SDS-PAGE to determine their protein composition.

Once the initial investigations into the composition of superoxide-generating fractions had been carried out, those fractions of interest were collected from subsequent chromatography runs, frozen and pooled, prior to being concentrated using an Amicon, N_2 pressurized, stirred, ultra-filtration cell. The membrane used in all instances was a YM 10 membrane, which had a molecular weight cut-off of 10,000kDa. Concentrated samples were then subjected to the BCA protein assay (Pierce) in order to determine protein concentration, and subjected to SDS-PAGE.

6.2.10 Protein elution

Bands on the IEF and non-denaturing gels that corresponded to stained bands exhibiting superoxide-dependent NBT staining, were excised from the gels and then subjected to one of two protein elution techniques, as described below. Individual protein bands, as resolved by SDS-PAGE, from the affinity chromatography fractions, were subjected to the latter elution technique, i.e. electro-elution.

Manual elution

This elution technique simply involved passive diffusion of the protein from the gel into an elution buffer. The elution buffer used was based upon the electrode buffer employed for the electrophoresis. Briefly, the gel slices to be eluted were thoroughly homogenised with 1ml of elution buffer in a 3ml, Uniform glass homogenizer (Jencons Ltd., Leighton Buzzard, UK). The homogenates were placed in a 1.5ml microfuge tube, the homogenizer was then rinsed out with 0.5ml of elution buffer, and the washings added to the microfuge tube. The tubes were vortexed well and placed on a rotating wheel overnight, at 4°C.

The resulting homogenates were spun at 13,000rpm for 10 minutes and the supernatants removed and retained. A further 500µl of elution buffer was placed in each tube containing the gel pellets. The tubes were vortexed well, and incubated on ice for 1 hour, vortexing occasionally. The tubes were spun once more, at 13,000rpm, for 10 minutes and these, second, supernatants were added to the first. The resulting eluents were stored at -20°C, or concentrated for further analysis.

Electro-elution

Protein was electro-eluted from gel slices using a GE 200 SixPac Gel Eluter from Hoeffer (Hoeffer Scientific Instruments, San Francisco, CA, USA), according to the manufacturers instructions. as follows. The elution buffers used were based upon the electrode buffer used in SDS-PAGE, at 1X and 4X concentrations. A water cooling system was attached to the apparatus and electro-elution performed at a current of 100V for 2 hours, with a reverse polarity, at the end of the 2 hours, lasting 5 seconds. The eluted protein, present in the 4X elution buffer, was stored at -20°C, or concentrated for further analyses.

6.2.11 SDS-PAGE

Prior to SDS-PAGE of eluted protein samples, the samples were concentrated using Centricon micro-concentrators (Amicon) with a molecular weight cut-off of 10,000Da. The dilute samples were placed into the concentrators and spun at 14,000rpm, at room temperature, for 30 minutes. The protein content of the concentrates were then determined via the BCA protein estimation method described in the general materials and methods section of this thesis. Appropriate volumes of the concentrates, containing 5µg of protein, were then diluted 1:1 with SDS-sample buffer and boiled for 5 minutes. The samples were stored at 4°C until the SDS-PAGE was performed.

SDS-PAGE was performed as previously described with 10% or 7.5% polyacrylamide gels, and with Rainbow™ molecular weight standards (Amersham) or with See-blue molecular weight standards (Novex). After electrophoresis the gels were silver stained according to the usual protocol.

6.3 Results

6.3.1 Solubilization and extraction of ROS generating ability from human spermatozoa

All of the detergents tested successfully extracted NADPH-dependent superoxide generating activity, as shown by superoxide-dependent NBT reduction, from human spermatozoa, with varying efficiencies. Extraction of ROS generating activity was shown by the ability of the protein extracts to reduce NBT to diformazan when supplied with NADPH (Figure 6. 1). The optimum detergent for extraction of ROS generating activity was n-octyl-ß-D-thioglucoside (OTG) and the least efficient was 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS). The different buffers used in the extraction buffers also differed in the extent to which they were able to support the extraction of ROS generating activity. The buffer most efficient in

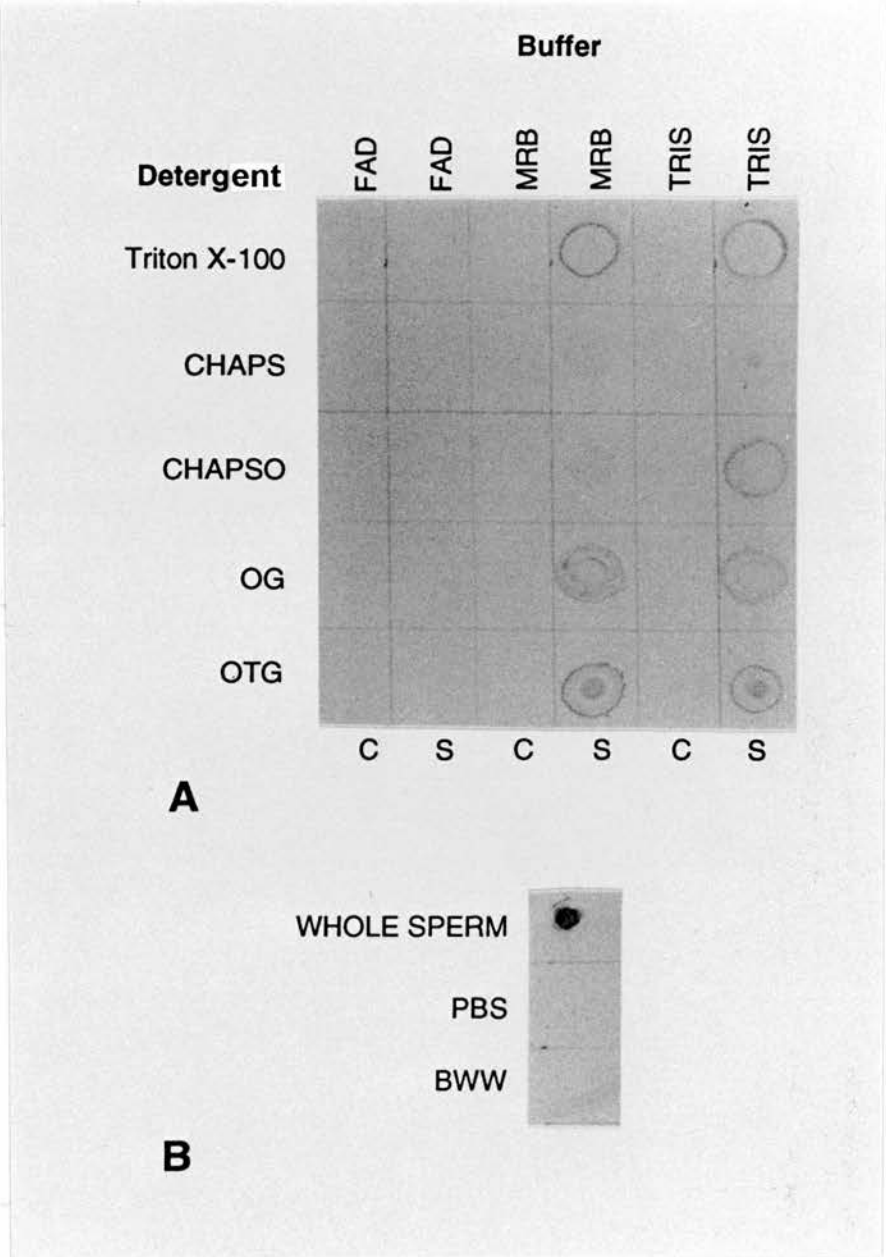

Figure 6.1 Dot blots of detergent-solubilized human sperm proteins and assessment of NADPH-dependent superoxide generating activity using the superoxide-dependent, NBT-based, staining protocol. Panel A compares the efficacies of various detergents and buffers in extracting superoxide generating activity and panel B shows the superoxide generating activity of whole sperm in PBS, PBS and BWW controls. Columns marked C do not contain any sperm protein, and those marked S, and the PBS/sperm dot, contain solubilized sperm protein from the equivalent of 10 x 10^6 spermatozoa/dot. The Tris-based buffer was the most efficient in terms of supporting the extraction of superoxide generating activity, and the most effective detergent was OTG. For abbreviations see text.

this respect was the Tris-based buffer. The MRB buffer also supported the extraction of ROS generating activity, but the FAD buffer was totally unable to do so. None of the extraction buffers tested were able to extract ROS generating activity from spermatozoa in the absence of detergent. In all subsequent experiments, the Tris-OTG extraction buffer was employed to extract the ROS generating activity from human spermatozoa.

6.3.2 Electrophoretic separation and isolation of sperm proteins capable of superoxide generation

Isoelectric focusing of human sperm OTG extract

Human sperm OTG extracts, containing the equivalent of 100 x 10^6 spermatozoa, were subjected to isoelectric focusing and then stained for superoxide generating activity employing the superoxide-dependent NBT staining protocol or stained for total protein with Coomassie Brilliant blue. Staining of IEF gels by the NBT-based method revealed 4 individual protein bands capable of ROS generation (Figure 6.2). The pIs' of these proteins were calculated by comparison to IEF standards and were shown to be approximately 7.63, 7.39, 6.73 and 6.52. By comparing the NBT staining pattern to the Coomassie blue stained IEF gel, it can be seen that the ROS generating components present in human spermatozoa are not particularly abundantly expressed proteins.

Attempts at eluting protein from the IEF gels were unsuccessful, whether employing the manual protein elution technique or the electro-elution method.

Non-denaturing PAGE of human sperm OTG extracts

200µl of human sperm OTG extract, ≈ $100x10^6$ spermatozoa, was used per gel lane, and following electrophoresis the gels were stained for ROS generating activity or for total protein. The ROS generating activity of human spermatozoa was resolved into discrete bands using this technique, although

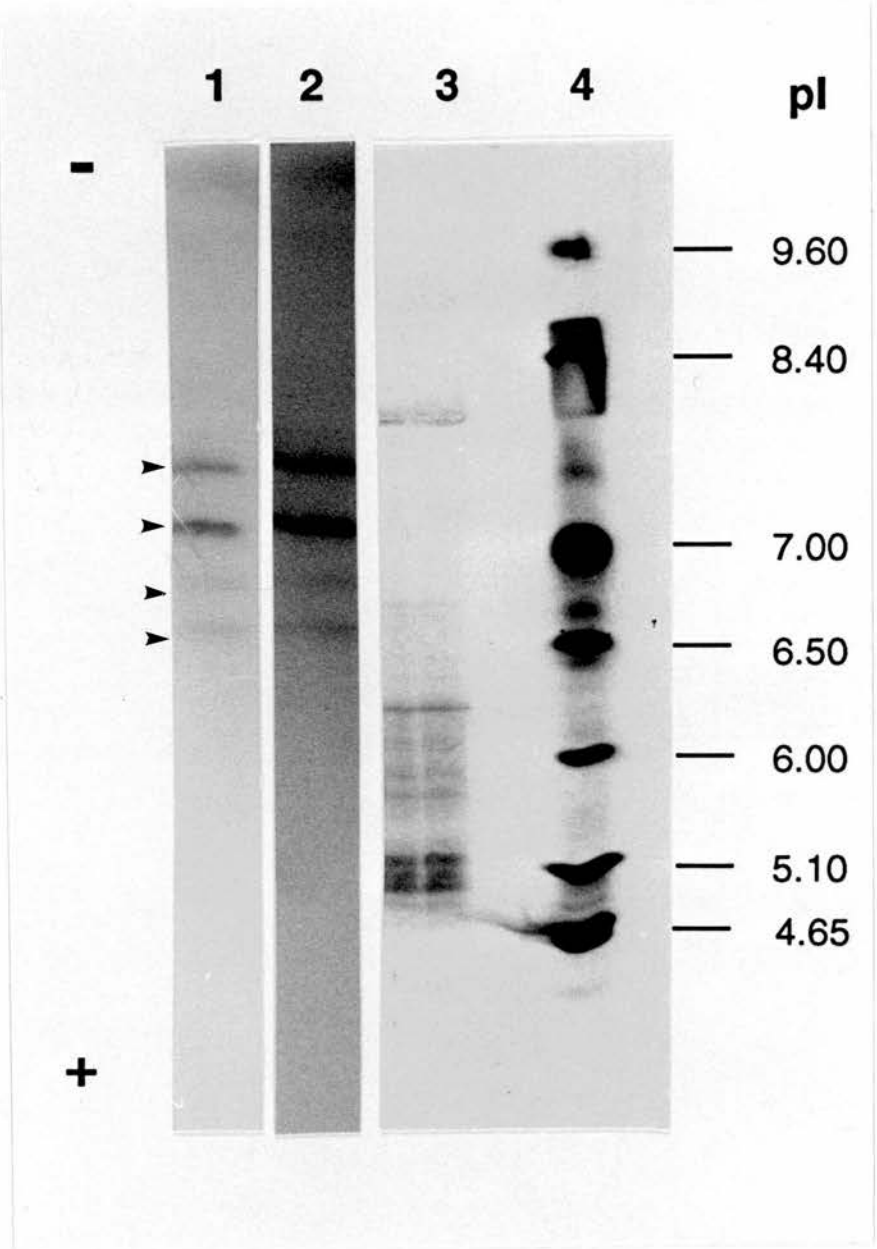

Figure 6.2 Iso-electric focusing of OTG-solubilized human sperm proteins. The panel shows the Coomassie blue stained, isoelectric profile of OTG-solubilized human spermatozoa (lane 3), and the superoxide-dependent, NBT staining pattern of the same solubilized sperm protein preparation (lanes 1 and 2). Solubilized sperm protein from 10 x 10^7 spermatozoa was used per lane. Lane 4 contains Coomassie blue stained, isoelectric point standards. Four individual protein bands were detected capable of superoxide anion generation, with pI's of approximately 7.63, 7.39, 6.73 and 6.52.

the bands were not as sharp as in the IEF gels (Figure 6.3). A further difference between the non-denaturing PAGE separation compared with the IEF separation was that the non-denaturing PAGE of sperm OTG extracts resulted in the identification of up to 6 protein bands capable of superoxide anion generation. Figure 6.3 shows a comparison of the NBT staining patterns of sperm OTG extracts from different donors and shows that although similar bands are stained in the different sperm extracts, the intensity of staining of the bands differed from donor to donor.

The dependency of the NBT staining protocol on the superoxide anion was confirmed by the inclusion of 1mg/ml SOD in the staining solution. This almost completely abolished the NBT staining, as shown in lane 4 of figure 6.3, although the bands showed differing susceptibilities to SOD-inhibition of staining. Comparison of NBT stained gels with a silver stained gel of human sperm extract (lane 5) resolved employing non-denaturing PAGE confirmed the observation made when employing IEF, i.e. that the proteins responsible for ROS generation by human spermatozoa are not very abundant.

Comparison of the superoxide-dependent NBT staining, with LDH-dependent staining of human sperm protein extracts revealed definite differences in the staining patterns (Figure 6.4, lanes 1 and 2). LDH-dependent staining revealed 4 separate, discrete bands, stained with varying intensities, the band corresponding to LDH C_4, staining the most intensely. Comparing superoxide dependent staining with total LDH and LDH C_4-dependent staining (Figure 6.4, lanes 1 and 2, and 3 and 4 respectively) shows that one of the superoxide generating bands, i.e. the band nearest the top of the gel, is very close to the position of the LDH C_4 band, possibly indicating that they are one and the same, although LDH is a NADH-specific enzyme, so this is probably not the case. However, the major superoxide -dependent NBT stained band is, quite definitely, not one of the LDH isoforms shown here.

Diaphorase-dependent MTT staining revealed two bands on the non-

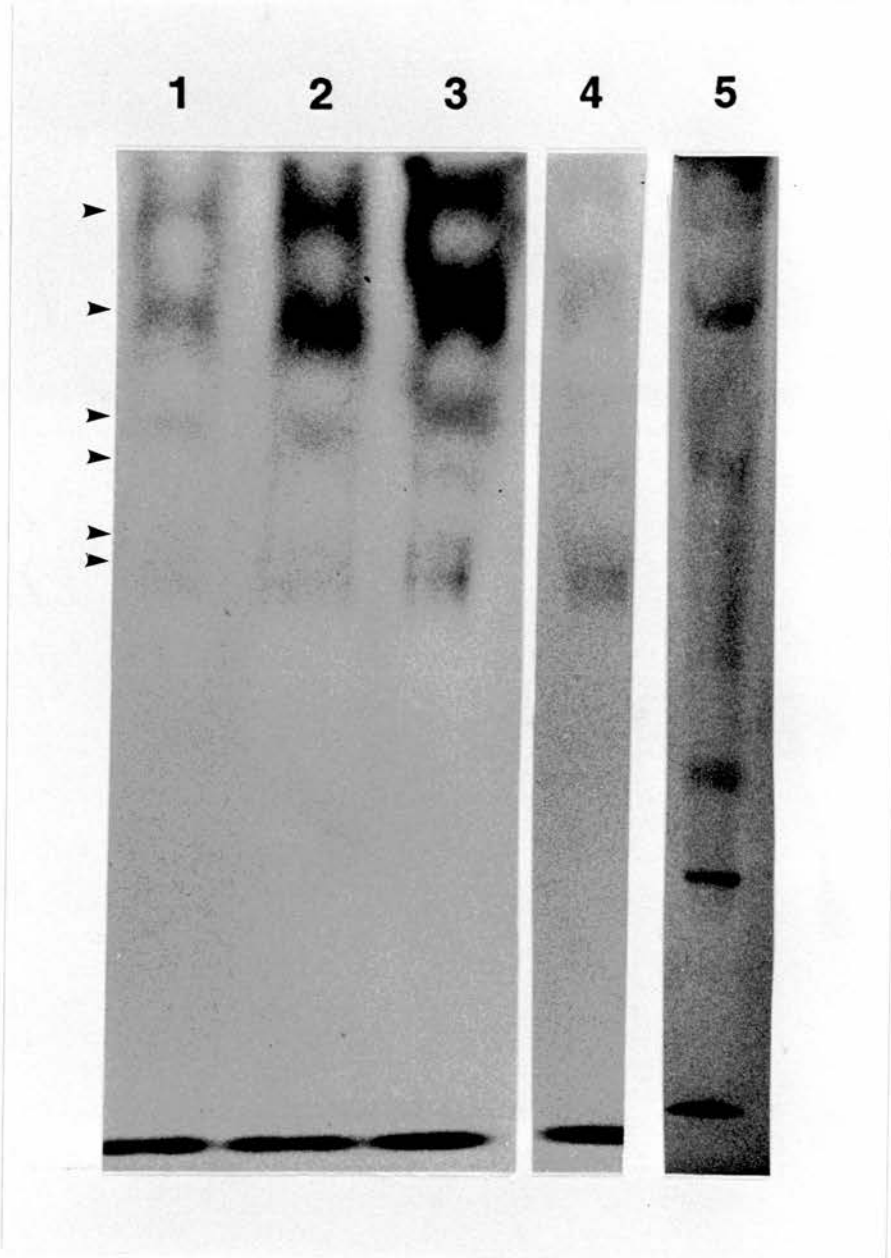

Figure 6.3 Non-denaturing PAGE of OTG-solubilized human sperm protein: comparison of NBT-staining profile of solubilized sperm protein from different donors. Lanes 1-3 contain OTG-solubilized protein from 10 x 10^7 spermatozoa, from 3 different, individual donors. 6 NBT-stained bands were identified, and the dependency of the staining upon the superoxide anion was confirmed through the inclusion of superoxide dismutase (1mg/ml) in the staining solution (lane 4). Inclusion of SOD almost completely inhibited staining. The sperm protein in lane 4 was from the same preparation as that in lane 3. Lane 5 shows a silver stain of the electrophoresed, OTG-solubilized, sperm protein

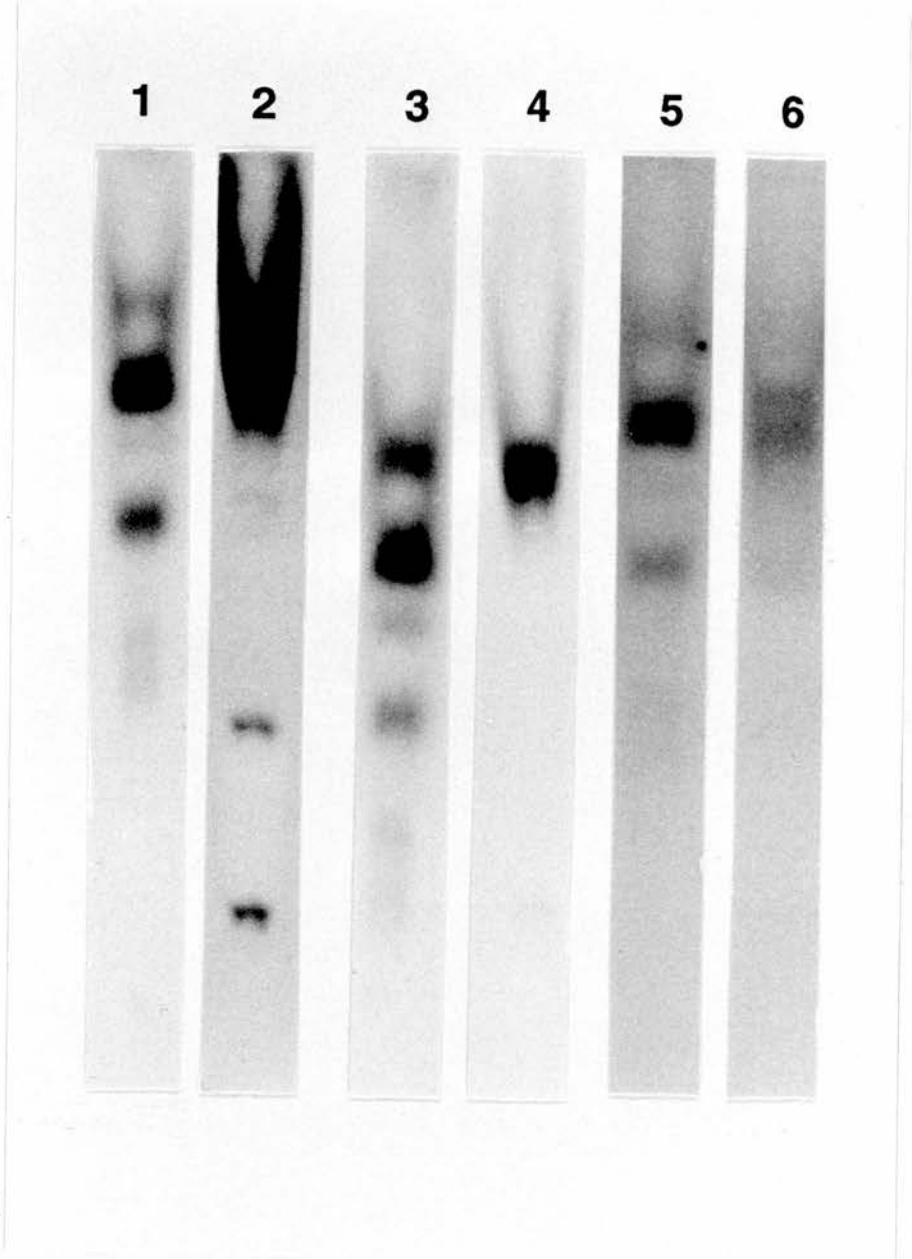

Figure 6.4 Non-denaturing PAGE of OTG-solubilized human sperm protein: comparison of various staining protocols. All lanes contain protein from 10 x 10^7 spermatozoa. Lanes 1, 3, and 5 were all subjected to the superoxide-dependent, NBT staining protocol. Lanes 2, 4, and 6 were subjected to lactate dehydrogenase, lactate dehydrogenase C_4 and diaphorase - specific staining protocols respectively. The protein profiles revealed by each staining protocol differed markedly from one another.

denaturing PAGE gels of sperm OTG extracts (Figure 6.4, lane 6). The diaphorase bands exhibited similar electrophoretic mobilities to the major superoxide-dependent NBT stained band, possibly indicating that this molecule/complex has some diaphorase activity (Figure 6.4, lanes 5 and 6).

Electro-elution, as with IEF gels, failed to elute any proteins from the non-denaturing PAGE gels, but, fortunately, manual elution was successful. Manually eluting protein from strips of gel corresponding to the 6 bands capable of superoxide generation, resulted in protein from the first 4 bands being successfully eluted, though no protein was retrieved from the last 2 bands. The bands successfully eluted, 1-4 in descending order with regard to gel position, were dissected into the following number of gel strips; bands 1 and 2, 3 strips and bands 3 and 4, 2 strips each. The protein eluates were then subsequently concentrated and resolved into their individual components by SDS-PAGE under non-reducing conditions.

SDS-PAGE of the protein eluates revealed them to be very heterogeneous protein populations, each consisting of numerous proteins (Figure 6.5).

6.3.3 2', 5' ADP affinity chromatographic separation and isolation of sperm proteins capable of superoxide generation

Affinity chromatography of human sperm OTG extracts, using 2',5' ADP-linked agarose, resulted in the differential separation of sperm proteins capable of superoxide anion generation (Figure 6.6). Superoxide generating activity was detected in 3 fraction groups. The first group of fractions corresponded to the void volume of the column, i.e. fractions containing protein that had not bound to the agarose and which eluted from the column rapidly with the application of HEPES-glycol buffer. The void fractions generated levels of superoxide anion that were in proportion to the amount of protein present in them. Column fractions that were eluted with buffer containing NADPH, and thus containing protein that had been bound to the

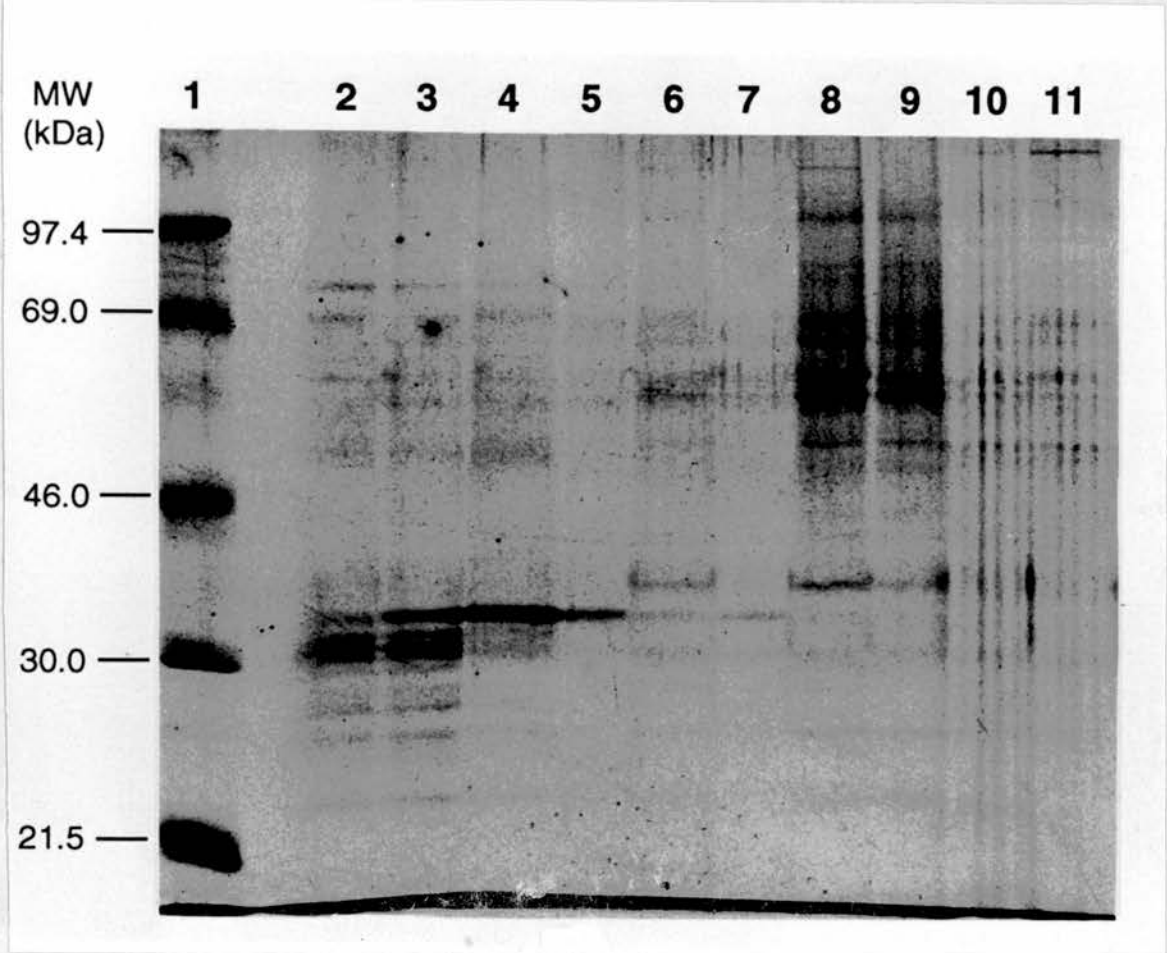

Figure 6.5 Representative, silver stained SDS PAGE gel of protein eluted from bands shown to be capable of superoxide anion generation via non-denaturing PAGE and NBT staining. Lane 1 contains molecular weight markers; lanes 2-4, protein from NBT stained band 1: lanes 5-7, protein eluted from NBT stained band 2; lanes 8-9, protein eluted from NBT stained band 3; and lanes 10-11 protein eluted from NBT stained band 4. For further details see text.

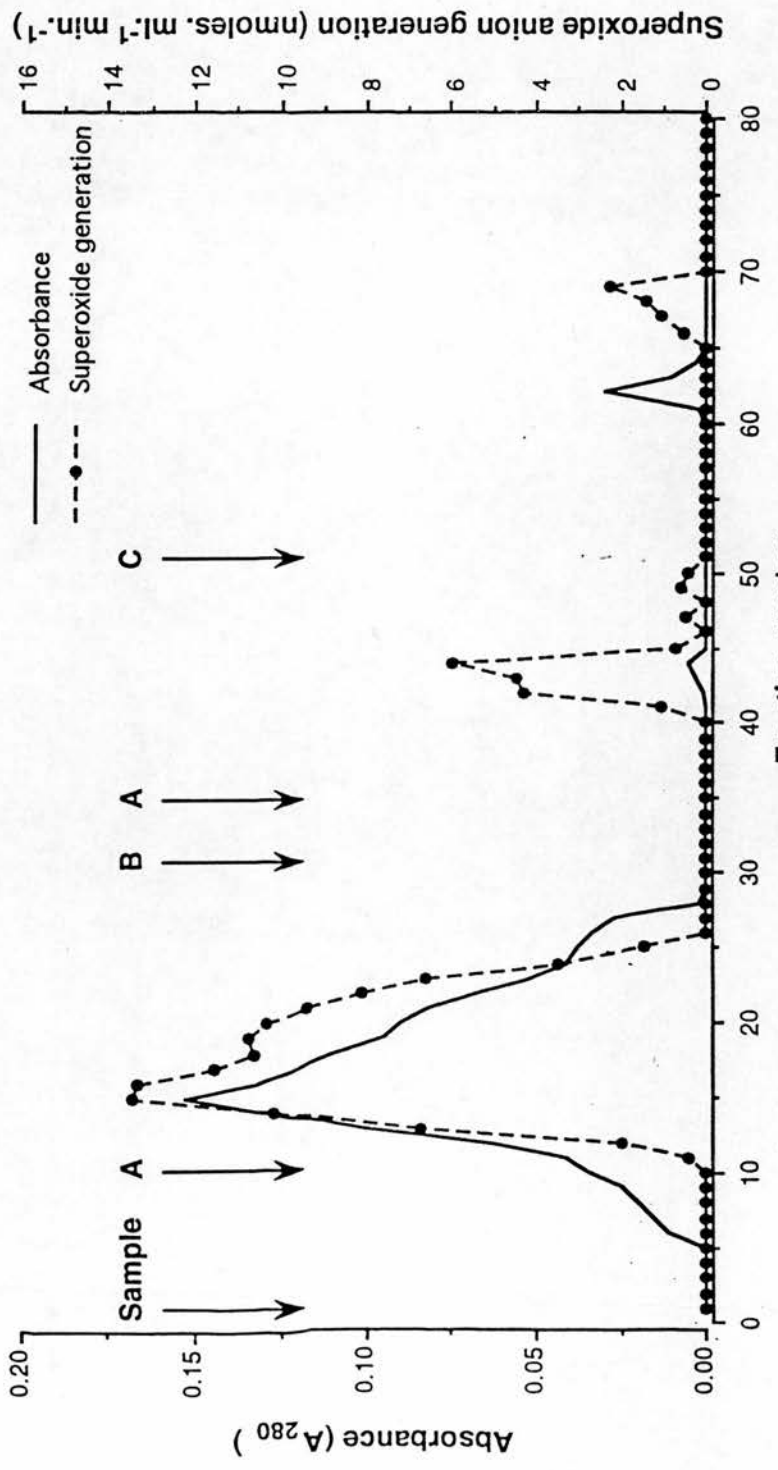

Figure 6.6 Representative elution profile of OTG-solubilized sperm protein extracts subjected to 2',5' ADP affinity chromatography. The arrows represent the various buffers applied to chromatographic column. The first arrow represents the application of the protein sample to the column; arrow A represents application of the HEPES-glycol column buffer; B represents the elution buffer, HEPES-glycol, 5mM NADPH; and C represents the regeneration buffer, HEPES-glycol, 4M NaCl. Fractions eluted from the column were assayed for superoxide generating activity via the NBT method, and the activity of each fraction is depicted, along with the absorbance, at 280nM, of each fraction.

2',5' ADP and subsequently displaced from the agarose with NADPH, showed levels of superoxide anion generation that were in excess of the levels that would have been expected had superoxide anion generation been a simple function of protein content. Fractions from the column that were eluted following the application of regeneration buffer containing 1.5M NaCl, also showed superoxide anion generating abilities, which were again in proportion to the amount of protein these fractions contained.

Figure 6.7 shows a silver-stained gel of the various affinity column fractions subjected to SDS-PAGE in a 10% gel. It can be seen that protein eluted in the void volume (lane 2) resolved into a large number of protein species, revealing the very heterogenic nature of the protein population contained in these fractions. The NADPH-eluted fractions contained considerably fewer proteins, with the majority containing only two significant protein bands, at around 55kDa and 80kDa. The NADPH-eluted fraction capable of maximal superoxide generation (lane 4), also only contained these 2 major protein bands.

In the first instance, it was decided to purify only one of these bands further, and hence the protein band migrating at 55kDa was extracted from the gel via electro-elution. Figure 6.8 shows the protein composition of samples at various points in the purification process, and Figure 6.9 show the final pure, 55kDa protein. The faint band in lane 3 above the 55kDa protein was shown to be a contaminant in the sample buffer used to prepare the 55kDa protein for electrophoresis, and was not present in the actual protein sample. In order to purify reasonable quantities of the 55kDa protein, the 4 fractions with greatest superoxide generating ability from numerous chromatographic runs were pooled, concentrated and then resolved into individual proteins via SDS-PAGE on 7.5% preparative gels, and the 55kDa band electro-eluted and then concentrated as before. The protein purified in this way was then stored at -20°C, in PBS, for use in subsequent studies.

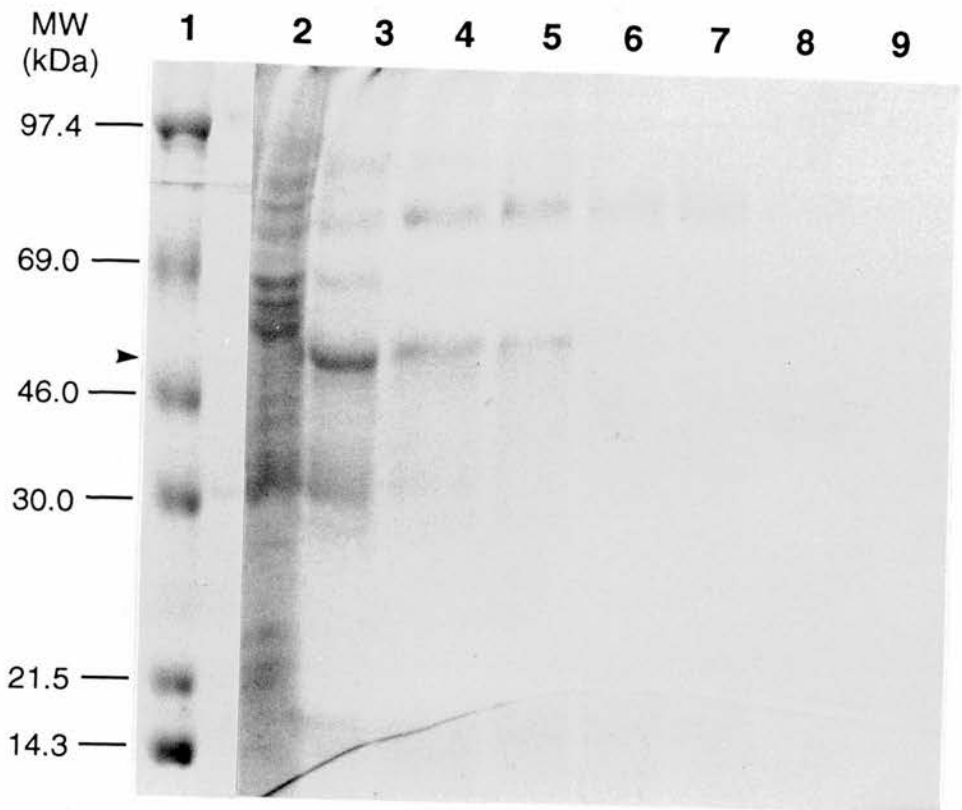

Figure 6.7 Representative silver stained SDS PAGE gel of various protein fractions from the 2',5' ADP, affinity chromatography. Lane 1 contains molecular weight markers. Lanes 2-9 contain protein from various fractions from the affinity chromatography. Lane 2, contains a void fraction, equivalent to fraction 15 on the previous figure; lanes 3-9 contain NADPH-eluted fractions, equivalent to fractions 41-47 respectively, of the previous figure (Figure 6.6). The arrow marks the most abundant NADPH-eluted protein, which migrated on SDS PAGE at around 55kDa. The other major, NADPH-eluted protein showed an apparent molecular weight of 80kDa.

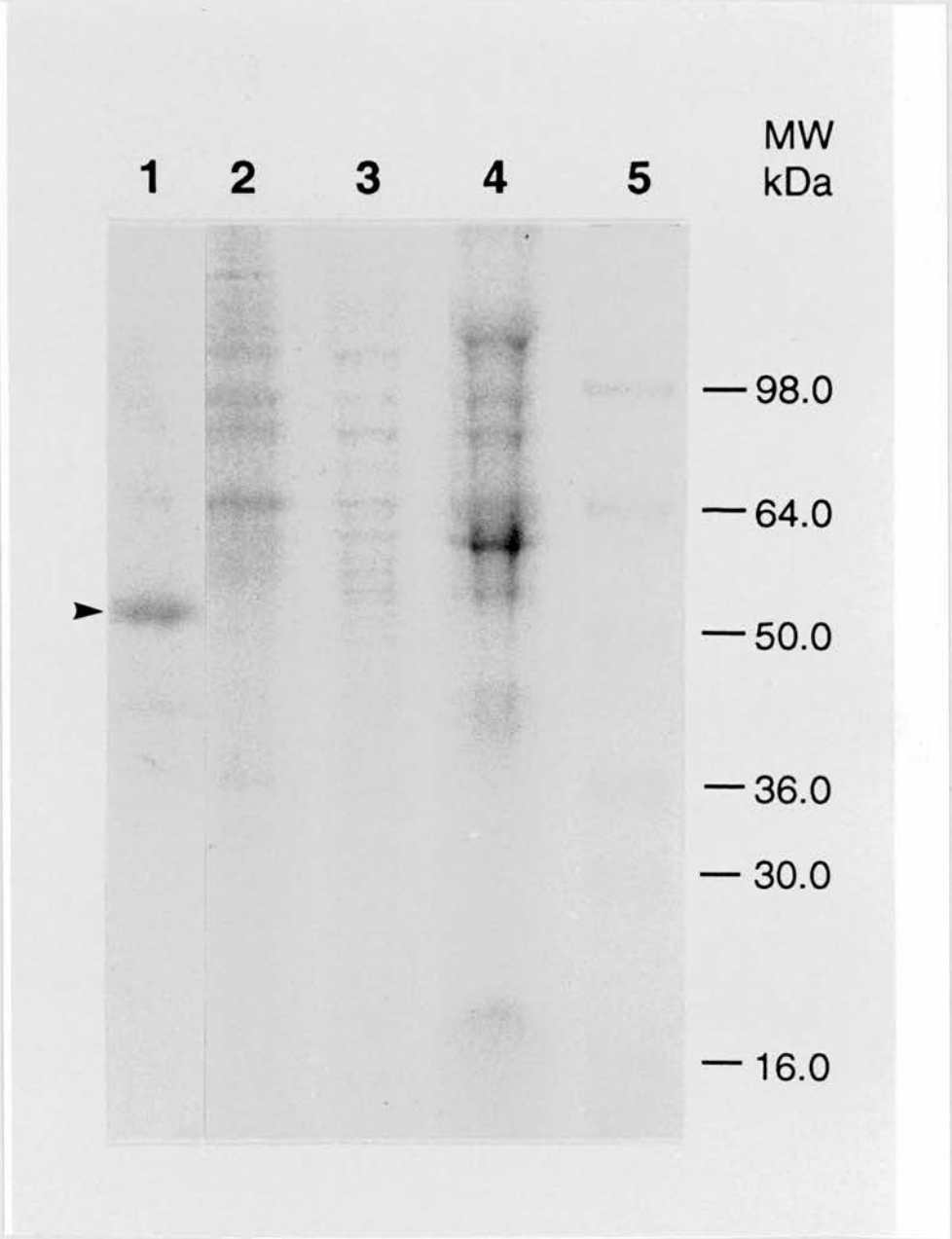

Figure 6.8 Representative silver stained SDS PAGE gel showing the protein profiles at various points in the purification of the 55kDa protein, putatively identified as a component of the human sperm superoxide generating oxidase. Lane 1 contains protein from an NADPH-eluted affinity chromatography fraction (the 55kDa protein is indicated by an arrow); lane 2, a chromatography void fraction; lane 3, OTG-solubilized human sperm protein; and 4, SDS-solubilized human sperm protein. Lanes 1-4 all contained the same amount of protein, i.e. 10μg. Lane 5 contains molecular weight makers.

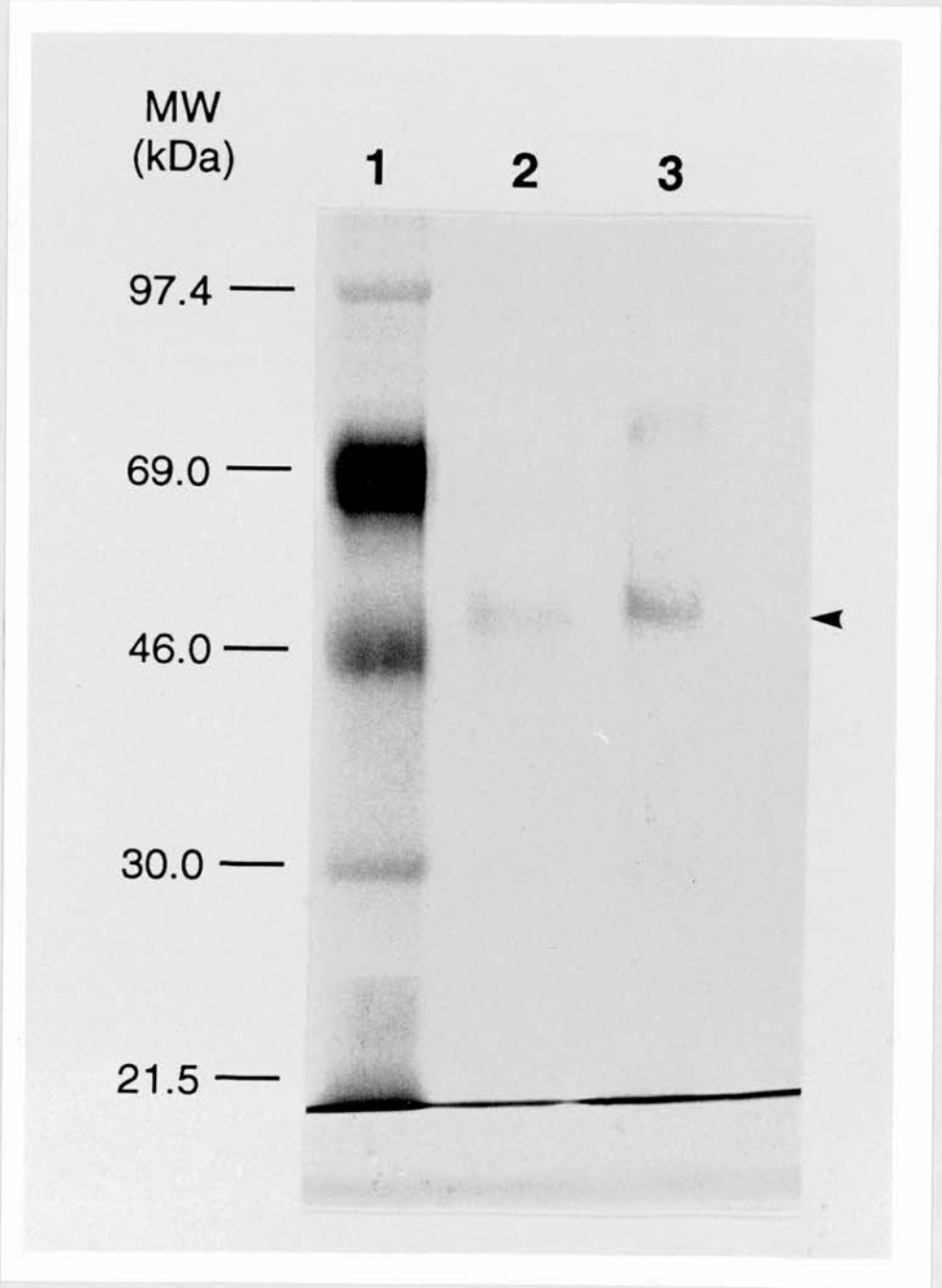

Figure 6.9 Representative silver stained SDS PAGE gel of the purified, electro-eluted, 55kDa human sperm protein, putatively identified as a component of the human sperm superoxide generating oxidase. Lane 1 contains molecular weight markers; lane 2 purified 55kDa protein, under non-reducing conditions; and lane 3, the purified protein under reducing conditions. Lanes 2 and 3 contained 5µg of protein. The second band visible in lane 3 was a contaminant present in the sample buffer.

6.4 Discussion

The cellular components responsible for ROS generation by human spermatozoa were extracted using a detergent-based protocol employing the non-ionic octylpyranoside detergent, n-octyl-ß-D-thioglucoside (OTG). This detergent successfully solubilized the ROS generating activity from human spermatozoa while maintaining its biological integrity and function. This was not a surprising result as a close relation of this detergent, i.e. n-octyl-ß-D-glucoside, has previously been used to successfully solubilize the superoxide generating NADPH oxidase of phagocytic leucocytes (Parkos *et al*, 1987; Sumimoto *et al*, 1992). Parkos *et al* found that this detergent was able to solubilize almost 100% of the cytochrome b_{558} of the oxidase present in human phagocytic leucocyte membranes, whilst Sumimoto *et al* (1992), also successfully solubilized superoxide generating activity from porcine neutrophil membranes with this detergent. Another member of the octylpyranoside detergent family, heptylthioglucoside, has also been used with success in NADPH oxidase solubilization and purification (Fujii and Kakinuma, 1991).

Subsequent to solubilization of ROS generating activity, the OTG protein extracts were successfully resolved into apparently discrete protein bands by IEF and non-denaturing PAGE. Initial results employing IEF to separate the complex mixture of proteins in the OTG sperm extract revealed 4 bands capable of superoxide anion generation, with pI values ranging from around 6.5 to 7.3. In contrast to this, non-denaturing PAGE revealed up to 6 bands capable of ROS generation, though it must be noted that two of these bands were almost insignificant in comparison to the other 4. The discrepancies between the 2 separation techniques may be indicative of the greater resolving power of non-denaturing PAGE over IEF, since the former separates proteins on basis of mass as well as charge. A second possibility is that two of the ROS generating components of human spermatozoa may have become inactivated during the IEF procedure.

The fact that more than one band capable of ROS generation were present on both types of gel, raises the possibility that the enzymatic system responsible for ROS generation by human spermatozoa may be present in multiple forms, either multiple isoforms of the same enzymatic system or multiple, completely different enzyme systems. The existence of multiple isoforms of enzymatic complexes is not unusual. An enzyme that demonstrates such a property is lactate dehydrogenase (LDH) (E.C. 1.1.1.27). This enzyme is a tetramer that catalyzes the interconversion of lactate to pyruvate, regulating cell metabolism under both aerobic and anaerobic conditions, and exists as 5 different isozymes in somatic tissues, with an extra isozyme being exclusively present in the post-pubertal testis and spermatozoa, i.e. LDH C_4 (Blanco and Zinkman, 1963). LDH C_4 is, in fact, specifically and uniquely expressed in post-meiotic germ cells and spermatozoa (Hintz and Goldberg, 1977), where it is thought to facilitate the use of lactate produced by Sertoli cells under the influence of follicle stimulating hormone (FSH) and insulin modulation (Oonk and Grootegoed, 1987).

The distribution of the various LDH isozymes in somatic tissues is dependent upon the metabolic requirements of the tissues, i.e. the extent to which they rely on anaerobic and aerobic respiration. This is due to the fact that the 2 different sub-units of LDH (A and B) have different catalytic properties and thus make up tetramer isoforms with different biochemical properties and, thus, different physiological significance. LDH C_4 is similarly composed of four sub-units, but is made up of only one type of sub-unit, C, which is distinct from the sub-units A and B of the somatic forms. Somatic forms of LDH are present in the testis and spermatozoa, but LDH C_4 accounts for between 50% and 80% of the total LDH activity present in spermatozoa (Blanco and Zinkman, 1963; Casano *et al*, 1991). Interest in LDH C_4 has largely been due to its possible potential as a sperm-specific antigen for contraceptive vaccine development (Goldberg *et al*, 1981). Indeed, investigations into this

molecules' potential for contraception have lead to the accumulation of a great deal of information on the properties of this enzyme. LDH C_4 has been shown to be present in the cytoplasm of spermatozoa, the mitochondrial sheath, the post-acrosomal region, the neck, the mid-piece and the tail sheath (Wang *et al*, 1990) and is probably functioning as an electron shuttle between the cytosol of the spermatozoon and the mitochondrion (Montamat *et al*, 1988). Elevated levels of LDH C_4 have been shown to be associated with male infertility, especially that associated with oligozoospermia, ROS generation and excessive retention of cytoplasm (Orlando *et al*, 1988; Casano *et al*, 1991; Gavella and Lipovac, 1992; Gavella and Lipovac, 1993; Orlando *et al*, 1994).

Thus, human spermatozoa contain 6 possible isozymes of LDH (including LDH C_4), elevated levels of LDH have been associated with male infertility; and certain studies have linked elevated levels of this enzyme with increased levels of ROS generation (Gavella and Lipovac, 1993). This information, in light of the fact that LDH can, under certain circumstances, catalyze the formation of the superoxide anion (Bielski and Chan, 1973), suggests a role for this enzyme in the induction of superoxide generation by human spermatozoa. Interestingly, non-denaturing PAGE of human sperm OTG extracts did reveal 6 bands capable of superoxide anion generation when supplied with NADPH. The possibility that LDH has a role in NADPH-induced ROS generation by human spermatozoa was investigated in an earlier chapter of this thesis, and shown to probably not be the case as sodium oxamate, an inhibitor of LDH, failed to inhibit superoxide anion generation by human spermatozoa. Although LDH may not be involved in generating the superoxide-dependent signals detected by lucigenin-mediated chemiluminescence, it remained possible that LDH activity was being monitored in the electrophoretic analyses. To investigate this possibility, the superoxide-dependent NBT staining patterns of human sperm OTG extracts were compared to LDH-dependent staining patterns and with LDH C_4-

dependent staining. Total lactate dehydrogenase staining identified 4 major bands, one of which, the strongest stained, was LDH C_4. Two of the isoforms of LDH were apparently missing from this analysis, possibly indicating that they had not entered the gel, or that they had not been extracted. That, of all the LDH isoforms, LDH C_4 showed the greatest activity in human spermatozoa was to be expected since this molecule has previously been shown to be the most active LDH isozyme present in spermatozoa (Blanco and Zinkman, 1963; Casano *et al*, 1991). Only LDH C_4 was positionally related to any of the superoxide-dependent NBT stained bands, raising the possibility that at least one of the superoxide generating bands was, indeed LDH C_4. This gained further support from the specific, LDH C_4-dependent staining of human sperm OTG extracts. Again this staining method showed LDH C_4 to be very closely located to the first NBT-stained band.

Clues to the identity of the major sperm protein band capable of superoxide anion generation were provided by the studies comparing superoxide-dependent NBT staining with diaphorase-dependent MTT staining. Diaphorase dependent staining, though not very sharp in terms of contrast, revealed two protein bands in human sperm OTG extracts. Comparison of the staining patterns obtained with the diaphorase and superoxide staining showed that one of the diaphorase bands was possibly in the same location as the major superoxide generating band. Although previous work described in this thesis appeared to indicate that diaphorase was not involved in human sperm NADPH-induced superoxide anion generation, it must be noted that the previous conclusion was based merely on the observation that dicoumarol, a DT-diaphorase inhibitor, had no effect on the NADPH-induced ROS generation by human sperm. It is possible that the diaphorase activity in human sperm is not inhibited by dicoumarol or that, as mentioned before, the systems for ROS generation being investigated in this instance are dissimilar to those investigated previously, via luminometry.

That the superoxide generating system of human spermatozoa might possess diaphorase-like activity would not be inconsistent with its main role as an oxidase, since this has been shown to be the case for other enzymes involved in electron transfer reactions, e.g. the NADPH oxidase (Fujii and Kakinuma, 1991) and nitric oxide synthase (Hope *et al*, 1991). In fact, Fujii and Kakinuma report that the NADPH oxidase may be formed by a membrane-bound, NADPH cytochrome c reductase, which is converted into a superoxide generating oxidase upon stimulation, and that NADPH can act as such a stimulant. Fujii and Kakinuma propose that the NADPH oxidase fits a model that is based on diaphorase-oxidase transition, and their proposition is supported by work carried out by Cross *et al* (1994) that has also revealed the expression of diaphorase-like activity by the leucocyte NADPH oxidase. None of this is at all surprising, since many enzymes exhibit diaphorase-like activity, which probably is more accurately defined as a property of enzymes involved in electron transfer reactions rather than as a specific enzyme in its' own right. The word diaphorase was coined many years ago (Farber *et al*, 1956), and merely refers to the ability of enzymes with NAD(P)H-dependent oxidoreductase activity to reduce various chemical dyes and nitro compounds, and hence cause a colour change, e.g. reduce NBT (Lind *et al*, 1990).

A role for a diaphorase-like enzyme in ROS generation by human spermatozoa is not inconsistent with what is already known about diaphorase activity in human spermatozoa. Human spermatozoa contain 3 isoforms of diaphorase, the activities of which are elevated in the spermatozoa from infertile men (Gavella and Lipovac, 1992) The same authors, in another study, showed a strong positive correlation between the levels of ROS generated by spermatozoa and their diaphorase activity, and some authors have postulated that the majority of human sperm ROS generation is indeed associated with some form of diaphorase activity (Gavella and Lipovac, 1992; Gavella and Lipovac, 1993; de Lamirande and Gagnon, 1995). Also, recently, there have

been reports suggesting that the testis and epididymis of the rat possess a form of nitric oxide synthase (Burnette *et al,* 1994; Adams *et al,* 1992), itself once described as a diaphorase, e.g. neuronal NADPH diaphorase (Hope *et al,* 1991).

Elucidating the identity of the proteins responsible for the generation of the superoxide anion by these seemingly discrete NBT bands was the next aim of this thesis. Unfortunately the proteins resolved by IEF were unable to be recovered by elution, possibly because they had precipitated at their isoelectric points. However, proteins were successfully eluted from the non-denaturing PAGE gels, from areas corresponding to those capable of superoxide anion generation. Subsequent analyses of the proteins present in these bands revealed them to be of a very heterogeneous nature, indicating that the resolving power of non-denaturing PAGE, in this instance at least, was not great enough to result in the total isolation of the individual proteins or protein complexes responsible for the observed ROS generation. However SDS-PAGE of the eluted proteins did reveal some interesting results. The protein population of the first band, the one that was possibly LDH C_4, did contain a dominant protein band that migrated at around 35kDa, the molecular weight of the sub-units of LDH. Unfortunately this was by no means the only band present in the eluent. The presence of many other, less intense, bands meant it was not possible to determine the identity of the band(s) generating superoxide in response to NADPH. However, it would probably be possible to determine the role, if any, of LDH C_4 in human spermatozoa superoxide anion generation by performing Western blot analyses of non-denaturing PAGE gels, with an antibody raised against LDH C_4. Unfortunately such an antibody is not commercially available and attempts at securing a sample of the antibody proved unfruitful: thus such analyses could not be performed.

A similar situation existed with the proteins present in the other eluted samples, which presented as complex mixtures of different molecular weights

with no evidence of individual dominant bands that might have been associated with superoxide generating activity.

Unfortunately, it had to be concluded that non-denaturing PAGE, as a separation technique, could be taken no further and thus, another experimental technique was employed to isolate the human sperm proteins involved in superoxide anion generation. To this end, 2',5' ADP affinity chromatography was employed as this had been successfully used to isolate the NADPH-binding component of the phagocyte NADPH oxidase (Yea *et al*, 1990; Umei *et al*, 1991); a neuronal diaphorase and a form of nitric oxide synthase (Hope *et al*, 1991; Bredt and Synder, 1990). This method is based upon the fact that NADPH-dependent enzymes bind, reversibly, to the structural analogue of NADPH, 2',5' ADP, and can then be displaced from the 2',5' ADP upon addition of the enzymes native ligand, i.e. NADPH, to the system. This technique successfully resulted in the selective, NADPH-dependent elution of superoxide generating activity from human sperm OTG extracts. The fractions possessing greatest ROS generating activity appeared to have only two major proteins present, one at around 55kDa and one at around 80kDa. No 35kDa protein, representing the sub-units of LDH, was present in any of the NADPH-eluted fractions. This was not really surprising as LDH utilizes NADH not NADPH as enzyme cofactor, and 2',5' ADP is not a structural analogue of NADH; 2',5' AMP is the structural analogue of NADH and affinity chromatography utilizing this ligand has been used to purify the various isoforms of LDH (Biodelius and Mosbach, 1973). Another notable feature of the eluted fractions was the absence of any proteins present with molecular weights corresponding to those of the main components of the leucocyte NADPH oxidase.

The presence of putative sperm oxidase protein components of 55kDa and 80kDa was interesting for several reasons, the first of these being that a 55kDa protein has been shown to be associated with the NADPH oxidase of

phagocytic leucocytes (Tanaka *et al*, 1992). Tanaka *et al* purified this protein to homogeneity from the porcine phagocytic leucocyte NADPH oxidase and showed that addition of this protein to reconstituted NADPH oxidase resulted in elevated levels of superoxide generation, and the authors postulated that this 55kDa protein was, in fact, a cytosolic component of the porcine leucocyte NADPH oxidase.

A second, and perhaps more interesting piece of information, is that constitutively expressed, endothelial nitric oxide synthase (NOS), one of the isoforms of the NADPH-dependent enzyme responsible for nitric oxide generation, and which has diaphorase-like activity, has been shown to have a molecular weight of 135kDa (Garvey *et al*, 1994). Another, very recent study, has shown that another form of NOS, in this case purified from human platelets, is in part made up of a 80kDa component, and is dimeric (Muruganandam and Mutus, 1994). It is possible that the second component is a 55kDa protein, and that in most tissues, such as endothelial tissue, these sub-units are tightly bound together, whilst in some they dissociate quite easily, resulting in the separation of the 55kDa protein and the 80kDa protein. This postulation, along with the apparent diaphorase-like activity of the major superoxide generating component of human spermatozoa as resolved by non-denaturing PAGE, perhaps suggests that one of the enzymes involved in ROS generation, i.e. NADPH-induced superoxide generation, by human spermatozoa is a form of NOS, though this postulation is highly speculative at this point. However, it has been shown that in neurones, depleted of L-arginine, the native substrate of NOS, the superoxide anion is generated and this is associated with neuronal NOS activation, and it has been shown that the superoxide anion is, in fact, synthesised by NOS (Culcasi *et al*, 1994). Other evidence, though not directly supporting an involvement of NOS in superoxide anion generation by human spermatozoa, but definitely showing that a role for it cannot be categorically ruled out, is that it has recently been

shown that the NADPH oxidase inhibitor diphenylene iodonium (DPI), inhibits endothelial NOS activity as well (Wang *et al,* 1993). So, the results presented in the previous chapter of this thesis, showing the DPI-mediated inhibition of superoxide anion generation by human spermatozoa, may have been due to an inhibitory action of DPI on a NOS-like system as well as, or instead of, an inhibitory action on a NADPH oxidase like system present in human spermatozoa.

In order to finally and conclusively resolve the identity of at least one of the cellular components involved in ROS generation by human spermatozoa, the 55kDa protein was purified to homogeneity. Purification of the protein would allow its identification via either of the following two methods. The first is a direct method involving the direct amino acid sequencing of the protein and the second, an indirect method, initially involving the generation of an antibody against the protein and subsequent immuno-screening of a testis cDNA expression library.

The first method requires that a sufficient amount of protein be purified to enable NH_2 terminal amino acid sequencing (Edman degradation), initially around about 5µg being required. However, if the NH_2 terminus proves to have been chemically modified, i.e. blocked, then much greater quantities of the protein, probably up to 1mg, will have to be purified to enable amino acid sequencing of the peptides resulting from a limited tryptic digestion of the protein (Matsudaira, 1990). Some proteins are natively blocked, whilst many become so during their purification. One such purification step that regularly leads to the blocking of the NH_2 termini of proteins is SDS-PAGE. This is due to the fact that free radicals, generated during the polymerization of the acrylamide gel, can attack the protein and oxidatively modify its NH_2 terminus, resulting in blocking. Precautions can be taken to avoid this problem, e.g. inclusion of free radical scavengers in the gel buffers and electrode buffers used in the electrophoresis, but even when such precautions

are taken, N-terminal blocking is still a commonly occurring feature of proteins purified by protocols involving an SDS-PAGE step (Matsudaira, 1990). The 55kDa protein under consideration here is even more likely to be blocked than most other proteins, even if the SDS-PAGE steps were omitted, as the protein itself generates the superoxide anion, a property that was exploited in its purification. Therefore, it was anticipated that the 55kDa protein was very likely to be NH_2 terminally blocked and thus, reasonably large quantities would have to be purified in order to get a very short stretch of sequence data, 10 or 15 amino acids, pertaining to this protein.

An alternative approach in these circumstances would involve the raising of a polyclonal antibody against the 55kDa protein. Once in possession of the antibody immuno-screening of a cDNA expression library could be undertaken leading to the identification of cDNA clones encoding the protein and hence the generation of sequence data. In addition, the availability of an antibody would permit studies into the expression and distribution of the protein, via Western blotting and immuno-cytochemistry. could also be performed. Thus, the next step in the identification of the superoxide generating system of human spermatozoa was to raise a polyclonal antibody against the 55kDa protein, and this work forms the basis of the following chapter of this thesis.

6.5 Summary and conclusions

Superoxide generating activity was extracted from human spermatozoa employing a protein extraction buffer containing the non-denaturing, non-ionic detergent n-octyl-ß-D-thioglucoside (OTG). The protein extract was resolved electrophoretically and revealed up to six protein bands capable of superoxide anion generation, when supplied with NADPH.

Comparison of the superoxide-dependent staining pattern of human sperm OTG extracts with the staining patterns achieved with LDH and

diaphorase-dependent staining, revealed that one of the sperm components capable of superoxide anion generation was possibly LDH C_4, and that one, the most active component capable of superoxide anion generation, might have diaphorase-like activity.

2',5' ADP affinity chromatography of sperm OTG extracts and subsequent SDS-PAGE analyses, revealed that the NADPH-eluted fractions capable of superoxide anion generation, contained two dominant protein components of 55kDa and 80kDa. The 55kDa protein was purified to homogeneity on SDS-PAGE gels and used in subsequent studies to characterize the molecule and study its expression and cellular distribution.

Chapter 7

Polyclonal antibody production, purification and characterization of the anti-sp55^{sox} antibody and its' antigen, sp55^{sox}.

7.1 Introduction

The ability to produce biological probes, antibodies, that specifically bind to a particular protein has provided a powerful tool for the biologist and biochemist. Antibodies are both versatile and sensitive tools for detecting and localizing specific molecules, and enable simple studies into protein function, identity, expression and purification. In this chapter immunological techniques were exploited to investigate the expression, and cellular localization of the sp55^{sox} protein that appears to be involved in ROS generation by human spermatozoa. In order to create a suitable platform from which to discuss this work, a brief overview of basic immunology and the theory behind polyclonal antibody production will be provided, starting with a few very basic definitions.

The term 'polyclonal antibody' is defined as the total population of antibodies present in animal serum (Dunbar and Schwoebel, 1990). Antibodies are glycoproteins that are an element of the adaptive immune system of vertebrates. Their production is induced when an animals lymphoid system contacts immunogenic foreign molecules, and is part of the **immune response** of the animal. To understand fully the biological significance of antibodies it is important to firstly understand the fundamental, underlying principals of the immune system and the immune response. These are outlined, very briefly, below.

7.1.1 The immune system

The immune system consists of two components; an innate, non-specific, arm that is the first line of host defence against infectious, and foreign agents; and

an adaptive arm that is specific to particular foreign entities, and long lasting in its effects (Roitt *et al,* 1985). It is the adaptive arm that involves the production of antibodies. Both types of immunity are regulated by leucocytes or white blood cells. Phagocytes, derived from the myeloid lineage of pluriopotent stem cells, such as polymorphs, monocytes and macrophages are involved in the innate immune response, whilst lymphocytes, again derived from pluriopotent stem cells, but in this instance the lymphoid lineage, are involved in the adaptive immune response and enable phagocytes to act on and kill foreign particles/organisms that they would otherwise not have recognised.

The lymphocyte lineage can be further differentiated into 3 populations, i.e. T lymphocytes, B lymphocytes, and a third population known as null cells that are not really important to this discussion and therefore, will not be referred to further. T lymphocytes differentiate in the thymus (hence their name) and are responsible for cell-mediated immunity, i.e. recognition of, and reaction with, foreign antigens upon the surface of other host cells, and they also play a regulatory role in other immune responses including antibody-mediated immunity (Roitt *et al,* 1985). B lymphocytes differentiate in the foetal liver and spleen in all vertebrates, in the bone marrow of adult mammals, and in the *bursa of Fabricius* of avian species; B lymphocytes are the cells that produce antibodies. Although the two lymphocyte cell types have different functions and their mechanisms of action are dissimilar, they are morphologically indistinguishable prior to stimulation and are very similar with respect to the way in which they exert very specific responses to foreign entities.

7.1.2 Immune response

A single antibody represents the secretory product from a single, stimulated B lymphocyte and its cloned progeny. During B lymphocyte differentiation, each lymphocyte aquires a specific receptor for an individual antigenic determinant

(epitope) present on a immunogenic molecule (antigen). One antigen can have many epitopes, which can consist of particular amino acid sequences, be conformationally determined, or be formed by molecular structures present on the antigen that are the result of post-translational modification, e.g. glycosylation, phosphorylation, etc.

The epitope-specific receptor on the surface of the B lymphocyte is a modified form of the antibody that has been inserted into the plasma membrane of that B lymphocyte. Many B cells may recognise a particular antigen, due to the presence of many different epitopes present on the antigen, but an individual B cell will only secrete one antibody, that recognizes a single, specific epitope. This leads to the question, how is an immune response elicited which is of sufficient magnitude to defend the host against invading, foreign molecules and organisms; one B cell being unlikely to produce enough antibodies on its own to enable the destruction of the infectious agent. In fact, the response of a single B cell does result in sufficient antibody production to successfully defend it's host against infection, and is able to do so due to a process known as **clone selection**.

Clonal selection is the process by which the number of B cells generating antibodies against a particular epitope is dramatically increased. When an antigen binds to an epitope-specific receptor on a B cell, this stimulates the cell to proliferate and mature, leading to a vast expansion of the number of clones of that particular B cell, all secreting a specific antibody against a defined epitope. The B cell clones then terminally differentiate to form either antibody secreting cells (plasma cells) or memory cells. The latter are non-antibody secreting, long lived, cells that retain the epitope receptor on the surface, but are dormant until stimulated by their target antigen. Memory cells constitute the host surveillance system for a recurrence of a given infection, enabling the host to be primed, ready to act more rapidly on subsequent exposure to a particular antigen. If these cells are stimulated, like virgin B cells they go

through clonal expansion, producing antibody-secreting plasma cells and further memory cells. A similar recognition and clonal expansion scenario occurs with T lymphocytes, only without antibody secretion; T cell stimulation is essential to elicit a powerful immune response, as they are involved in regulating antibody production by the B lymphocytes (Harlow and Lane, 1988).

7.1.3 Antibody production and structure

On binding of antigen to a B cell, the latter is stimulated to produce the appropriate antibody. It does this by a dramatic increase in the number of membrane bound polyribosomes and the intense activity of the rough endoplasmic reticulum. The antibodies produced and secreted are identical to the receptor in the B cells, except at the extreme carboxyl terminus, where the membrane-bound form has a long string of hydrophobic amino acids which traverse the lipid bilayer of the membrane (Alberts *et al*, 1989). The secreted form has a much shorter string of water soluble amino acids. The switch from membrane-bound, to secreted antibody requires a different nucleotide sequence at the 3' end of the mRNA and this involves RNA spicing during production of the mRNA. The final exon of the DNA, encoding the antibody, contains the nucleotides coding for the hydrophobic carboxyl terminus, and the nucleotides, present in the intron preceding it, encode for the water soluble tail. Thus, by splicing out this intron, during mRNA production, the membrane-bound antibody is formed. A much shorter primary transcript, ending before the beginning of the last exon which contains the acceptor splice site, generates the secreted, water soluble antibody molecule (Early *et al*, 1980).

The antibody molecule itself is a glycoprotein, and its basic structure comprises four polypeptide chains, two identical light chains and two identical heavy chains (Figure 7.1), each of which contain constant and variable regions. The antibody is a bifunctional molecule binding to the infectious agent and to

the cells of the immune system. The variable, epitope binding region of the antibody is formed by the heavy and light chain variable regions at the amino termini. The two variable regions are closely associated and bound to one another by a combination of non-covalent interactions and disulphide bonds. The constant region of the antibody is the portion that binds to other components of the hosts immune system. Certain enzymes can split antibodies into fragments, e.g. papain splits an antibody molecule into two **Fab** fragments (**f**ragment-**a**ntigen-**b**inding), and one **Fc** fragment, the constant fragment (Roitt *et al*, 1985).

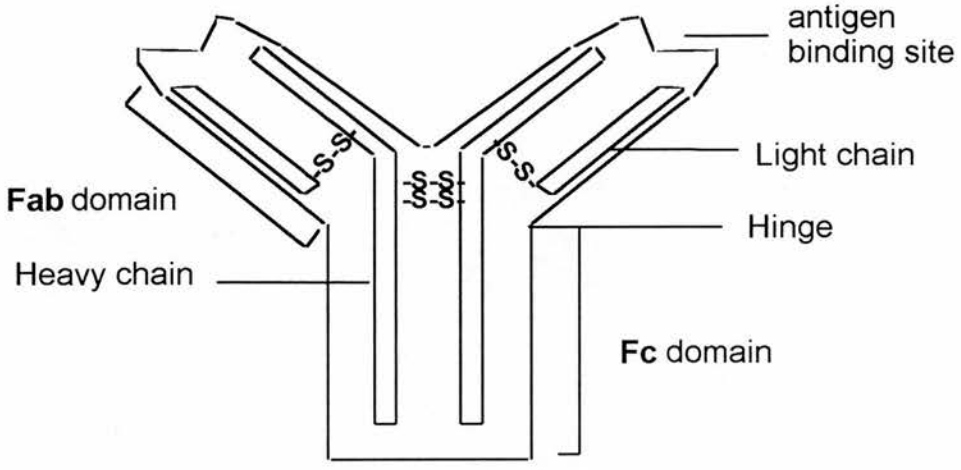

Figure 7.1 Simple diagrammatic representation of a typical antibody molecule.

Different classes of antibodies are formed due to the existence of different classes of heavy chain. The classes are IgA, IgD, IgE, IgG, and IgM. IgA antibodies exist as two sub-classes; IgA1 and IgA2, and IgG antibodies exist as four subclasses, IgG1, IgG2, IgG3, and IgG4. The various heavy chains impart a distinctive conformation to the hinge and tail regions of the antibody, and give each class (and subclass) characteristic properties (Roitt *et al*, 1985).

7.1.4 Antibody-antigen interaction

Antigens bind to antibodies through the epitope specific to that antibody, and are bound by reversible, non-covalent, bonds, e.g. hydrogen bonds,

electrostatic bonds, Van der Waals forces, and hydrophobic interactions. These types of bonds may be relatively weak individually, in comparison to covalent bonds, but together, they constitute considerable binding energy. The overall strength of the bonding is representative of the affinity of the antibody for the antigen.

Antibody-antigen binding is very specific for particular epitopes, but not always for particular antigens. This is because different antigens may share the same epitopes, and thus an antibody raised against antigens containing such epitopes, may not be very antigen-specific, and cross reactivity with other antigens may be observed. However, this aside, antibodies do show remarkable epitope-specificity and have the potential to differentiate readily between even very closely related epitopes.

7.1.5 Antibody response

When an animal encounters a foreign molecule, its immune response to it depends on the antigenicity of the molecule (its ability to provoke an immune response), and the form of its presentation to the immune system. The T and B lymphocytes must be able to recognise epitopes on the molecule and respond as outlined above. The antibody response can be classified into two types; the primary immune response, and the secondary immune response (Roitt *et al*, 1985). The primary response occurs when the immune system encounters a foreign molecule for the first time, and is characterized by an initial lag period during which no antibodies are produced, followed by a logarithmic rise in antibody production that reaches a plateau and then declines due to catabolism of the antibodies. The main class of antibody produced by the primary response are IgM antibodies. The secondary response occurs when the immune system encounters a foreign molecule for a second time. This response is characterized by a very short lag period before antibody production, an extended plateau period at an elevated level, and the main class

of antibody present is IgG. The secondary response is much more potent compared to the primary, due to the presence of memory cells formed during the initial response (Harlow and Lane, 1988).

Not all antigens are very immunogenic. This may be due to the antigens structure and the immune systems' ability to respond to it. However, the immunogenicity of an antigen can be increased by presenting it to the host in an adjuvant (Harlow and Lane, 1988). An adjuvant is a delivery system, usually a water in oil emulsion, that causes non-specific enhancement of the immune response. The use of an adjuvant produces an enhanced, and prolonged response to the antigen because of three main reasons. The first of these is that the use of an emulsion to carry the antigen, acts to protect the antigen from rapid catabolism. The adjuvant also acts as a depot, enabling prolonged antigen stimulation, via the slow and sustained release of antigen into the hosts circulation. The third way in which an adjuvant works to increase the immune response, is through the addition of a microbial antigen, e.g. the heat killed *Mycobacterium tuberculosis* in Freunds complete adjuvant. The addition of a microbial antigen enhances the production of antigen non-specific factors such as cytokines, that enhance the overall, specific immune response to the antigen of interest.

Finally, it should always be born in mind that the antibody response is a co-ordinated reaction of B cells, T cells, and antigen presenting cells, communicating directly or indirectly, with one another, via specific or non-specific immune factors. It is a complex series of events that can often be manipulated to serve the purposes of various research aims (Roitt *et al*, 1985).

7.1.6 Use of antibodies in the study of proteins

Many areas of biology and medicine have used antibodies in order to pursue the study of particular proteins (antigens) and this is particularly true in the field of gamete biology. Using antibodies, many sperm antigens, including

those important in fertilization, have been identified and their roles determined through antibody-inhibition studies, e.g. G-proteins (Garty *et al*, 1988), LDH C_4 (Goldberg, 1986), PH-20 (Lathrop *et al*, 1990), FA-2 (Naz *et al*, 1993). For a review of such studies see Myles (1993).

Antibodies can be used to determine the presence of an antigen in cells or tissue using biochemical (Western blotting, Towbin *et al*, 1979), and histological (immunocytochemistry and immunohistochemistry) techniques. Antibodies are also very useful in the purification of antigens, on a small or large scale, via immunoprecipitation and immunoaffinity chromatography (Harlow and Lane, 1988). Antibodies can also be used to determine the amount of a particular antigen present in a sample via the ELISA (enzyme-linked immunoassay) technique (Engvall and Perlmann, 1971), and very importantly, antibodies can be used in functional studies, to determine the precise role of a particular protein. Finally, antibodies can be used to identify cDNA clones from expression libraries that are generating the target antigen (Sambrook *et al*, 1989).

The work described in this chapter uses these some of these techniques in order to pursue the identification of the 55kDa human sperm protein, apparently involved in ROS generation by these cells. Antibodies against this molecule have also been used to enhance our understanding of the possible biological role of this sperm antigen, determine its cellular distribution and its tissue and species-specificity.

7.2 Materials and methods

7.2.1 Cell preparation

Human semen samples were prepared as previously described. In antigen purification, only the resulting sperm suspensions that were free of leucocyte contamination used. Animal spermatozoa were isolated from the

epididymides of the rat, guinea pig, mouse and hamster as described in Chapter 9 of this thesis.

7.2.2 Antigen purification

The 55kDa antigen, designated sp55^{sox}, was purified by a combination of 2',5' ADP affinity chromatography, as described in Chapter 6, and then the antigen was purified to homogeneity via SDS-PAGE and subsequent electroelution, again as described in Chapter 6 of this thesis.

Post-purification, protein concentration was estimated via the BCA method and then stored at -20°C until used for immunization.

7.2.3 Immunization

An adult female, New Zealand White rabbit was used to produce the polyclonal antibody, and was housed in the Centre for Reproduction animal house under normal conditions. All animal handling procedures, including performance of injections and bleeding, were carried out by the staff of the animal house, and their assistance is gratefully acknowledged.

The primary immunization was performed with pure antigen. The antigen was mixed 1:1 with Freunds Complete Adjuvant (FCS, Sigma) and a thick emulsion formed via homogenization. Once a thick emulsion had been formed, which did not disperse on the surface of PBS, it was drawn up into a 2.5ml disposable, plastic, sterile Plastipak syringe (Becton Dickinson, Madrid, Spain), and immediately used for immunization. The immunization was performed by sub-cutaneously injecting the antigen-adjuvant emulsion into the rabbit, at multiple sites.

The animal was given a booster injection 4 weeks later. This consisted of antigen mixed with Freunds Incomplete Adjuvant (FIA, Sigma). Again a thick emulsion was formed which was subsequently, sub-cutaneously injected into the rabbit. A second boost was given 8 weeks later.

Two weeks after each booster injection the rabbit was bled, and the serum separated from the red blood cells as follows. The blood was allowed to clot at 4°C overnight, then the clot was removed, and the remaining preparation centrifuged at 3,000 rpm for 30 minutes to pellet any remaining cells and insoluble debris. The supernatant was removed and stored at 4°C overnight, to allow any remaining insoluble material to settle out. This preparation was centrifuged once more at 3,000 rpm for 30 minutes, and the plasma supernatant removed and retained for IgG purification as outlined below. Any anti-serum not subjected to IgG purification was stored at -20°C.

7.2.4 Antibody purification

The IgG antibody fraction was purified from the polyclonal anti-serum employing Protein G affinity chromatography, using fast performance liquid chromatography (FPLC®) apparatus. A pre-packed, Protein G Superose® HR10/2 from Pharmacia (Pharmacia LKB Biotechnology) was used, the matrix being composed of Protein G covalently attached to a rigid, cross-linked agarose-based gel, Superose 12. The column was attached to FPLC apparatus from Pharmacia consisting of P-3 peristaltic and P-500 pumps, an LCC-500 liquid chromatography controller unit, a UV-M UV detector and chart recorder, and a Frac-300, fraction collector.

The crude anti-serum was sequentially passed through a series of filters prior to column application. The filters were of the following pore sizes; 1.5µm, 0.8µm. 0.45µm, and 0.22µm, and were all from Millipore (Millipore, Watford, UK.). Once filtered, 1.5 ml of the anti-serum was mixed 1:1 with column starting buffer, which consisted of 20mM Na_2HPO_4 titrated to pH 7.0 with 20mM NaH_2PO_4 (the resulting buffer contained roughly equal quantities of each). The filtered serum/buffer was then applied to the column using a 3ml, disposable syringe. The FPLC apparatus was programmed to run start buffer through the column until any non-bound antibody and serum proteins were

eluted. The IgG was eluted with a gradually increasing, continuous gradient of elution buffer, consisting of 0.1M glycine-HCl, pH 2.7. All buffers were run through the column at a flow rate of 1ml/min and 1ml fractions collected throughout. The eluted IgG was indicated by an obvious secondary absorbance peak on the UV chart-recorder, and the fractions corresponding to this second peak were pooled and stored on ice until they could be concentrated with the IgG containing fractions from other Protein G affinity FPLC runs.

The fractions containing the purified IgG antibodies were pooled and concentrated using an Amicon, stirred ultra filtration cell, model 8010 (Amicon), fitted with a YM 30 Diaflo membrane (Amicon), under a constant flow of nitrogen at a pressure of 35psi. The affinity fractions were concentrated to approximately 1ml and then made up to the original volume of pre-purified anti-serum, using sterile PBS (Flow). The concentrated IgG preparation was then assessed for protein concentration via the BCA method and stored at -20°C in 500µl aliquots, with the addition of 0.1% BSA (Sigma) and, to half of the aliquots, 0.05% sodium azide (Sigma).

7.2.5 Western blotting

The antigenic, tissue and species specificity of the anti-sp55^{sox} IgG was evaluated by the Western blot procedure employing non-denaturing PAGE and SDS-PAGE.

Sample preparation

For non-denaturing PAGE, human sperm samples were solubilized using OTG extraction buffer as described in the previous chapter of this thesis (Chapter 6). For NBT staining purposes, 100 x 10^6 spermatozoa were solubilized, and for Western blotting, 20 x 10^6 spermatozoa. Bromophenol blue at a concentration of 0.002% was added to the OTG extracts before they were applied to the gels.

For SDS-PAGE all sperm suspensions were washed X3 with Tris-washing buffer before protein extraction was carried out, as previously described. Human sperm samples were solubilized, again as previously described, using SDS sample buffer. 100µl of extraction buffer was used per 20 x 10^6 spermatozoa, and for each gel lane, a volume of sperm extract the equivalent of 5 x 10^6 spermatozoa, i.e. 25µl, was used. The spermatozoa of various animal species were extracted in a similar fashion with the same quantity of spermatozoa being used per gel lane.

After SDS-extraction the protein extracts were mixed 1:1 with either reduced or non-reduced SDS sample buffer and heated to 100°C for 5 minutes. Samples were allowed to cool before application to the gel, or stored at 4°C until use.

Either pre-stained Rainbow molecular weight markers (Amersham), as described earlier, or pre-stained SeeBlue™ molecular weight markers (Novex Experimental Technology, San Diego, CA, USA) were used. The SeeBlue™ markers consisted of the following pre-stained proteins: myosin, 250kDa; bovine serum albumin, 98kDa; glutamic dehydrogenase, 64kDa; alcohol dehydrogenase, 50kDa; carbonic anhydrase, 36kDa; myoglobin, 30kDa; lysozyme. 16kDa; aprotinin, 6kDa; and insulin, ß chain, 4kDa. 10µl of either of the markers were used per lane, mixed 1:1 with reduced sample buffer and then boiled for 5 minutes, before use.

Electrophoresis

SDS-PAGE was performed using 7.5% gels as previously described and non-denaturing PAGE was performed using 5% gels, again as previously described. Electrophoresis was performed at 30mA through the stacking gel and at 20mA through the resolving gels, until the sample dye front almost reached the bottom of the gel.

Post-electrophoresis, SDS-PAGE gels were blotted onto Hybond C super

nitrocellulose (Amersham) by the semi-dry method described earlier. Non-denaturing PAGE gels either stained for superoxide generating activity (previous chapter) or blotted onto nitro-cellulose using the Trans-Blot blotting apparatus (Bio-Rad), according to the manufacturers instructions, at 15V (constant), 500mA overnight. The transfer buffer in this instance was 25mM Tris, 192mM Glycine (both from Sigma), and 20% methanol (BDH).

Human multiple tissue blot

A commercially prepared, human multiple tissue blot was also used in the Western blot analyses conducted with the anti-sp55^{sox} IgG. The material used was a human MTW, 7721-1, blot from Clontech (Clontech Laboratories Inc., Palo Alto, CA, USA), and was composed of SDS-solubilized protein from brain, heart, kidney, lung, skeletal tissue, and liver. The electrophoresed proteins were blotted onto a PVDF membrane, and a total of 75µg of protein was present per lane. The blot was subjected to the same immunological detection protocol as the self-prepared blots, as described below.

Immunochemical detection

Post-blotting, nitrocellulose membranes and the PVDF multiple tissue blot, were subjected to the usual antibody-based, antigen detection protocol, with the following antibodies and modifications. Membranes were blocked as normal in 10% milk powder in TBS and then incubated in primary antibody overnight at 4°C. The primary antibody was anti-sp55^{sox} IgG, at a dilution of 1 in 100. The secondary antibody used was donkey anti-rabbit IgG, HRP conjugate (Amersham), and was used at a dilution of 1 in 6000: membranes were incubated in this antibody for 1 hour at room temperature.

After incubation with the secondary antibody, the nitrocellulose membranes were subjected to the usual washing procedure and then processed for antigen detection using the enhanced chemiluminescent Western blotting

detection kit from Amersham. This procedure was carried out according to the manufacturers instructions, as already outlined in the materials and methods chapter of this thesis (Chapter 3).

7.2.6 Immunofluorescent localization of sp55^{sox} on human and rat spermatozoa

Indirect, immunofluorescent staining was carried out on human and rat spermatozoa in order to determine the cellular localization of the sp55^{sox} molecule. The immunological basis of this technique is illustrated in Figure 7.2, and was carried out as described below.

Preparation of spermatozoa

100% and 50% human sperm suspensions were washed 3X in PBS prior to use for immunocytochemistry, in order to remove any albuminar from the cells and their medium. The washing procedure involved centrifuging aliquots of spermatozoa at 500g for 5 mins, resuspending in PBS and then repeating this process 2 further times. The cells were finally resuspended, in PBS, at a concentration of 5×10^6/ml and used immediately.

Rat spermatozoa were isolated from the cauda and caput epididymides as described previously. Suspensions were subjected to the washing protocol described above and finally resuspended at a concentration of 1×10^6/ml in PBS.

Slide preparation

Slides were prepared for immunocytochemistry by spreading 10µl of the various sperm suspensions on each well of a 12 well Henley slide (CA Henley, Essex, UK). The slides were allowed to dry overnight, at room temperature, or for 2 hours at 37°C, before use. Slides not being used immediately were individually wrapped in foil and stored at -70°C until required. Prior to use for immunocytochemistry, the frozen slides were thawed at room temperature

for 30 mins before being unwrapped.

Cell fixation

All slides were subjected to paraformaldehyde fixation. This fixation method maintains an intact cell plasma membrane, and retains surface antigens in an unaltered state. Paraformaldehyde fixation was performed by immersing the cell coated slides in 1% paraformaldehyde (Sigma) in PBS (Flow) in a staining dish, on a gently rocking platform for 10 minutes. The slides were then washed X2 in distilled water for 2 minutes per wash, again in a staining dish on the rocking platform. Prior to staining all slides were washed in TBS for 20 minutes.

Immunofluorescent staining

All antibody incubations were carried out in a humid box, created by placing water saturated paper towels in the bottom of a sealable, polythene container.

Prior to incubation with primary antibody all slides were placed in the humid box and blocked with normal goat serum (NGS) (Sigma) diluted 1 in 10 with TBS. 10µl of the 1/10 NGS was applied to each well of the Henley slides. After 20 minutes excess NGS was wiped away from between the wells and the 10µl of control rabbit serum (CRS) or the primary antibody, i.e. anti-sp55^{sox} IgG, applied to the wells. The CRS and primary antibody were used at dilutions ranging from neat, to 1 in 25. The slides were then incubated for around 1 hour 30 minutes in the humid box, at room temperature.

The slides were then washed 3 times in TBS for 5 minutes per wash and then returned to the humid box. At this point, 10µl of the secondary antibody, i.e. affinity purified goat anti-rabbit IgG, FITC conjugate (Sigma), at a dilution of 1 in 50 was applied to each well of the Henley slides. The slides were then incubated for two hours in the humid box at room temperature, protected from light. After 2 hours, the slides were washed twice in distilled water for 2

minutes per wash, and stored in a black slide box, protected from light and drying out, until use.

The stained slides were mounted in Citifluor (Citifluor UKC, Canterbury, UK), an anti-fluorescence quenching reagent, viewed using fluorescence microscopy and photographic records made.

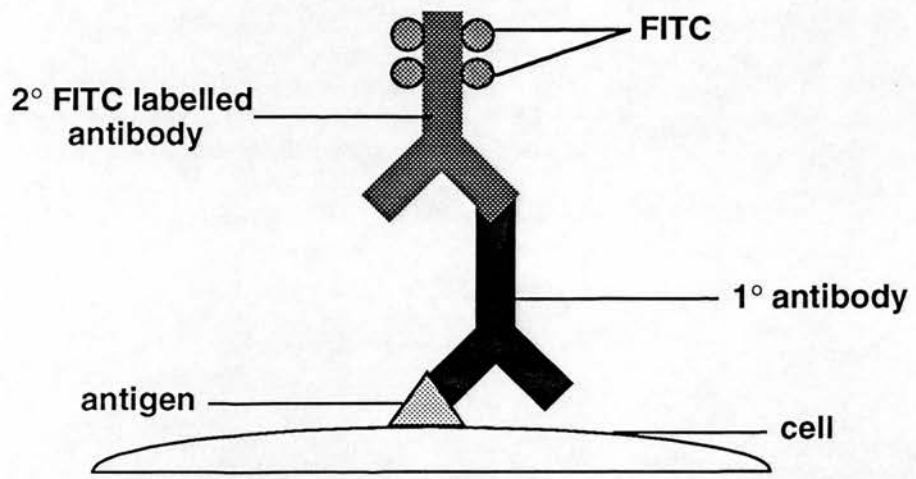

Fig 7.2 Diagrammatic representation of indirect, immunofluorescent staining. The secondary antibody, conjugated to fluorescein isothiocyanate (FITC), is raised against the IgG of the species in which the primary antibody was raised.

7.2.7 Effect of the anti-sp55^{sox} IgG on NADPH-induced superoxide anion generation by human spermatozoa.

The possibility that the anti-sp55^{sox} IgG would inhibit superoxide anion generation was investigated. This involved incubating intact sperm suspensions and OTG solubilized sperm preparations, with the IgG, inducing superoxide anion generation by the addition of NADPH, and then monitoring the resulting superoxide anion generation. Briefly, 400µl aliquots of a leucocyte free, 100% sperm suspension at 10 x 10^6 cells/ml, were incubated with 16µl of the IgG, 16µl of CRS, or 16µl of BWW, and then superoxide anion generation was monitored via lucigenin-dependent chemiluminescence. Once a steady basal signal had been obtained, superoxide anion generation was

stimulated with the addition of 500µM NADPH, and the subsequent lucigenin-dependent chemiluminescence monitored for around 20 minutes. The same protocol was followed with the OTG sperm extracts. In this instance 100µl of OTG sperm extract (equivalent to 10 x 10^8 cells/ml), was added to 300µl of BWW, and the effects of the IgG and CRS on NADPH-induced superoxide anion generation determined as outlined above.

In both instances the experiments were performed in triplicate and the results analysed as follows. The integrated chemiluminescent signal over two five minute periods were calculated, i.e. immediately before and after NADPH addition. The NADPH-dependent chemiluminescence was then calculated by subtracting the number of chemiluminescent counts calculated for the 5 minute period prior to NADPH addition, from the total number of chemiluminescent counts calculated in the five minute period after NADPH addition. The results were expressed as the mean chemiluminescence of three separate experiments (counts / 5 mins).

7.3 Results

7.3.1 Antigen purification and subsequent immunization

The 55kDa protein, designated $sp55^{sox}$, was successfully purified to homogeneity. The mean yield of the purification process was approximately 25.2ng of the 55kDa protein per 10^6 spermatozoa. Three rounds of purification were carried out to produce the antigen for immunization, and these are summarised in Table 7.1. Each round of purification involved 4 individual affinity chromatography runs, 1 SDS-PAGE purification step, and 1 electro-elution step. The primary and secondary immunizations were performed with 100µg of antigen, whilst in the final boost, only 32µg was used. The immunization schedule is outlined in Table 7.2.

Table 7.1 Antigen purification schedule.

Purification round	Affinity chromatography (mean number spermatozoa/run)	Total sperm protein	Total recovered protein (55kDa)
1	1385×10^6	1.252g	134.6µg
2	1255×10^6	1.134g	200.0µg
3	919.5×10^6	0.623g	32.0µg

Table 7.2 Immunization schedule.

Immunization	Time	Quantity	Adjuvant	Route
1°	week 0	100µg	Freunds complete	sub-cutaneous
1st boost	week 4	100µg	Freunds incomplete	sub-cutaneous
2nd boost	week 12	32µg	Freunds incomplete	sub-cutaneous

The rabbit was bled 1 week after the first boost and 1 week after the second. The anti-serum from the first bleed contained 6.44mg/ml protein and the anti-serum from the second bleed contained 6.76mg/ml.

7.3.2 IgG purification

The IgG antibody fraction was purified from the crude polyclonal anti-serum by Protein G affinity FPLC, and a representative trace from the FPLC is shown in Figure 7.3, showing the absorbance at 280nm (A_{280}). The column eluates containing the purified IgG were concentrated, the total volume made up to

the original serum volume with sterile PBS, and the protein concentration of each IgG solution determined. The anti-serum from the first and second bleeds contained similar amounts of IgG, giving concentrations of 5.32mg/ml and 4.6mg/ml protein respectively.

7.3.3 Western blot analyses with the anti-sp55^{sox} IgG

In order to characterize the anti-sp55^{sox} antibody and its corresponding antigen, sp55^{sox}, Western blot analyses were carried out. The purified IgG specifically cross reacted, on blots from non-denaturing PAGE, with a single band, that corresponded to one of the NBT-stained protein bands, although the quality of immunological detection was consistently very poor. Figure 7.4 shows a representative non-denaturing PAGE blot.

On blots from SDS-PAGE the IgG gave much cleaner results. The IgG consistently and specifically cross-reacted with a band which migrated at approximately 55kDa. In about 50% of cases the IgG also detected a band at 135kDa, that was sometimes the predominant band recognised. The level of antigen expression detected by the anti-sp55^{sox} IgG showed donor to donor variation (Figure 7.5a) and also variation within individual donors, between the sperm Percoll® fractions used (Figure 7.5b). The general pattern of expression was that sperm protein extracts from the 50% Percoll® fractions showed elevated levels of expression, compared to the spermatozoa from the 100% fraction.

Western blotting under reducing conditions did show marked differences from that carried out under non-reducing conditions (Figure 7.6). When sperm proteins were subjected to SDS-PAGE under reducing conditions, an overall increase in the level of detection of the 55kDa and 135kDa bands was observed.

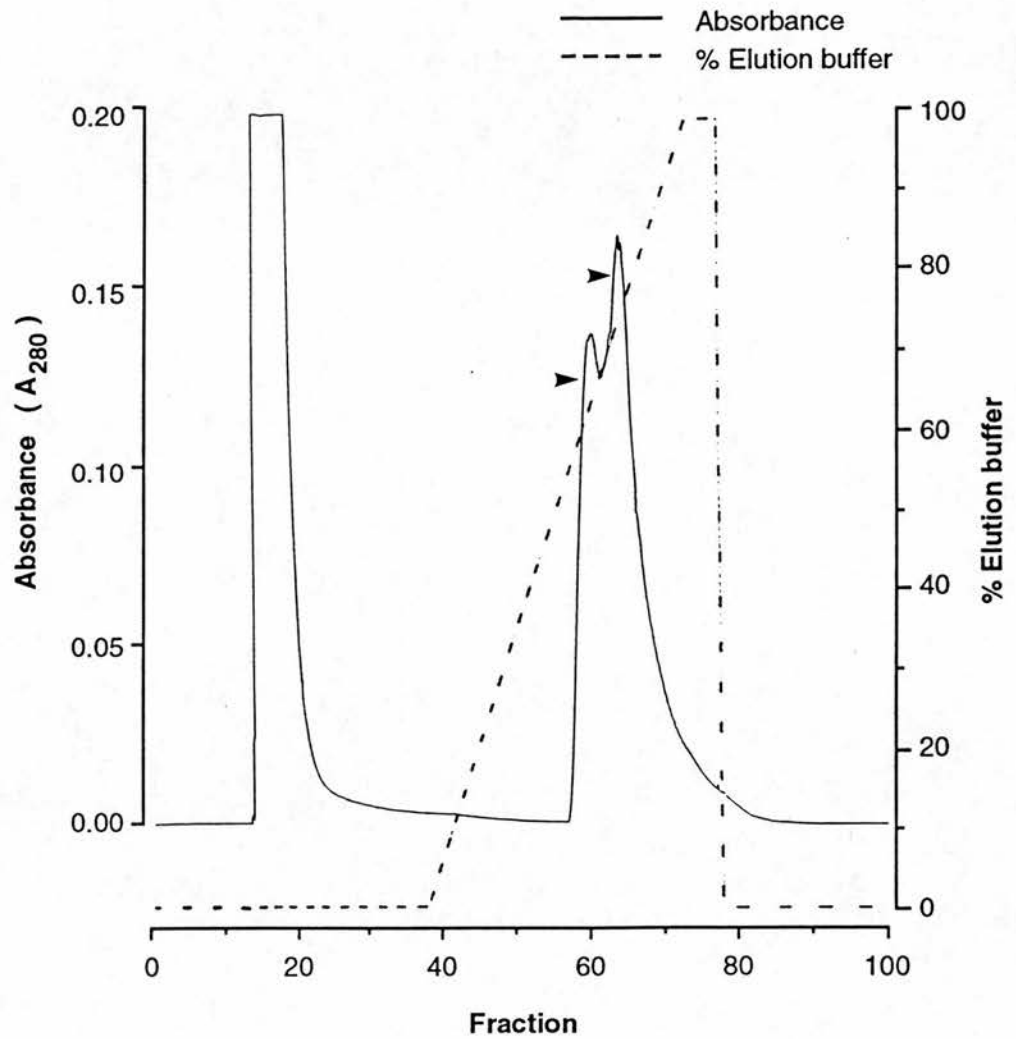

Figure 7.3 Representative elution profile of the Protein -G affinity FPLC of the anti-serum from the rabbit immunized with the 55kDa, putative sperm oxidase component. The first peak represents the elution of crude serum proteins and non-IgG antibodies. The second peak represents the elution of the IgG fraction of the anti-serum and is composed of 2 composite peaks, probably indicative of different IgG sub-classes.

Figure 7.4 Comparison between NBT staining, and Western blotting of OTG-solubilized human sperm proteins subjected to non-denaturing PAGE. Lane 1, containing approximately 10 x 10^7 spermatozoa, was NBT stained. Lane 2 and 3, containing the equivalent of 1 x 10^7 spermatozoa from the same sample as lane 1, were Western blotted and probed with the anti-sp55^{sox} IgG and rabbit pre-immune serum respectively. The arrow indicates the NBT stained band that corresponds to the single band recognized by the anti-sp55^{sox} IgG.

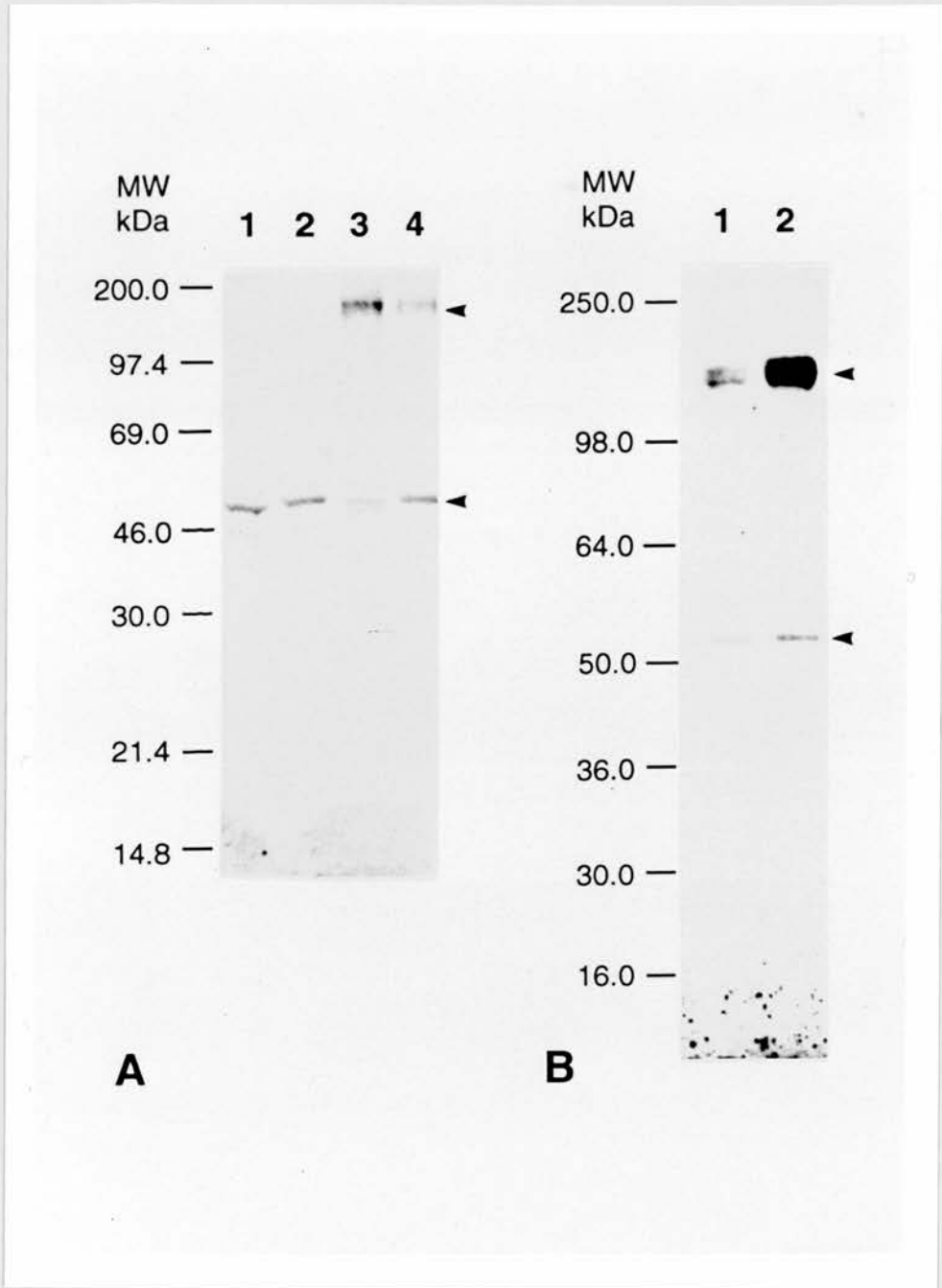

Figure 7.5 Representative Western blot of SDS-solubilized human sperm proteins subjected to non-reducing SDS PAGE, showing the donor to donor variation, and sperm intra-sample variation, in the expression of antigen recognized by the anti-sp55^{sox} IgG. Panel A: lanes 1-4 each contain solubilized sperm protein from a different donor. Panel B; lane 1 contains solubilized sperm from the 100% fraction of a sperm sample, and lane 2 contains sperm from the 50% fraction of the same sperm sample. On both panels the anti-sp55^{sox} IgG detected two bands, one at 55kDa and one at around 135kDa, as indicated by the arrows. The equivalent of 5 x 10^6 spermatozoa were used per lane. The position of the molecular weight markers are indicated to the right of each panel.

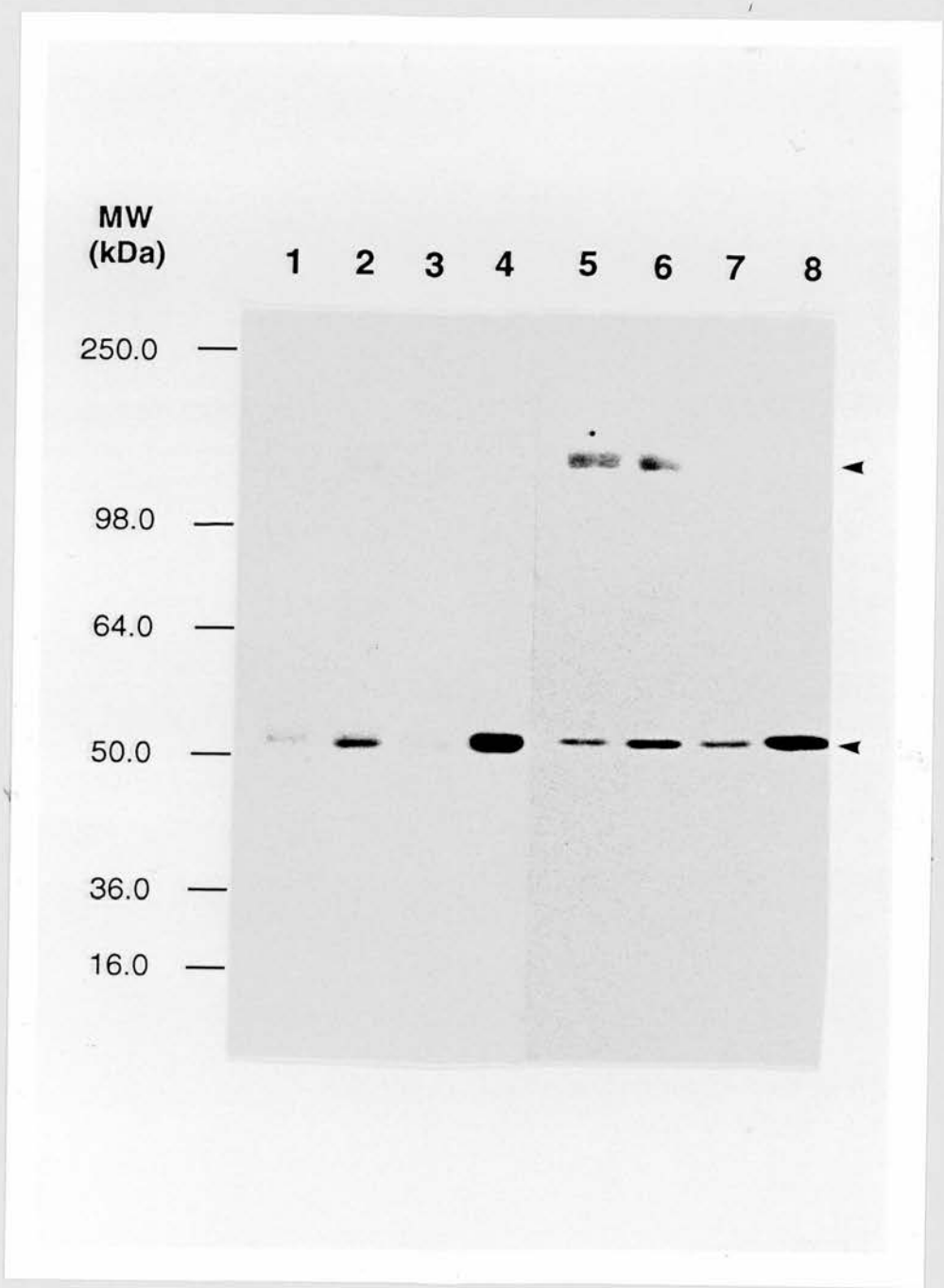

Figure 7.6 Representative Western blot of SDS-solubilized human sperm proteins subjected to SDS PAGE under reducing and non-reducing conditions, and then probed with the anti-sp55^{sox} IgG. Lanes 1-4 were electrophoresed under non-reducing conditions and lanes 5-8 electrophoresed under reducing conditions. The different individual samples used in lanes 1-4 matched those used in lanes 5-8. As before the anti-sp55^{sox} IgG detected two bands one at 55kDa and one at 135kDa (arrows). Each lane contained the equivalent of 5x 10^6 spermatozoa and the positions of the molecular weight markers are indicated to the left of the panel.

In some instances, when the 135kDa protein was not detected in sperm extracts resolved under non-reducing conditions, electrophoresis of the same protein sample under reducing conditions, lead to its detection of the 135kDa protein on Western blots.

The anti-sp55^{sox} IgG cross-reacted with sperm protein extracts from the rat, but not with the sperm protein extracts from the mouse, hamster or guinea pig (Figure 7.7). Figure 7.7 shows that the antibody cross-reacts with a rat protein that is slightly larger than the corresponding protein in human spermatozoa, i.e. approximately 58kDa. A few minor bands are also apparent. The same figure further illustrates the donor to donor variation observed in the expression of the 55kDa protein (lanes 1-4).

To further explore the expression of the sp55^{sox} antigen by rat spermatozoa, Western blots, constructed using protein extracts from cauda, corpus and caput rat epididymal spermatozoa, were probed with the antibody. The results of this are shown in Figure 7.8. The antibody cross reacted with a band at approximately 58kDa in the cauda and corpus spermatozoa and cross reacted with this band, and an additional band migrating at approximately 55kDa, in the caput spermatozoa.

The anti-sp55^{sox} IgG was shown not to be exclusively reactive with sperm proteins. Figure 7.9 shows a multiple human tissue Western blot probed with the anti-sp55^{sox} IgG. The antibody recognised 3 proteins of different molecular weights on this blot. These were a 50kDa protein, present in brain, heart, and skeletal tissue; a 55kDa protein present in liver and kidney only; and a 65kDa protein that was present, at various levels, in all the tissues examined here.

7.3.4 Immunofluorescent localization of sp55^{sox} on human and rat spermatozoa

The regional localization of the sp55^{sox} antigen was determined employing immunofluorescent staining of paraformaldehyde fixed spermatozoa from

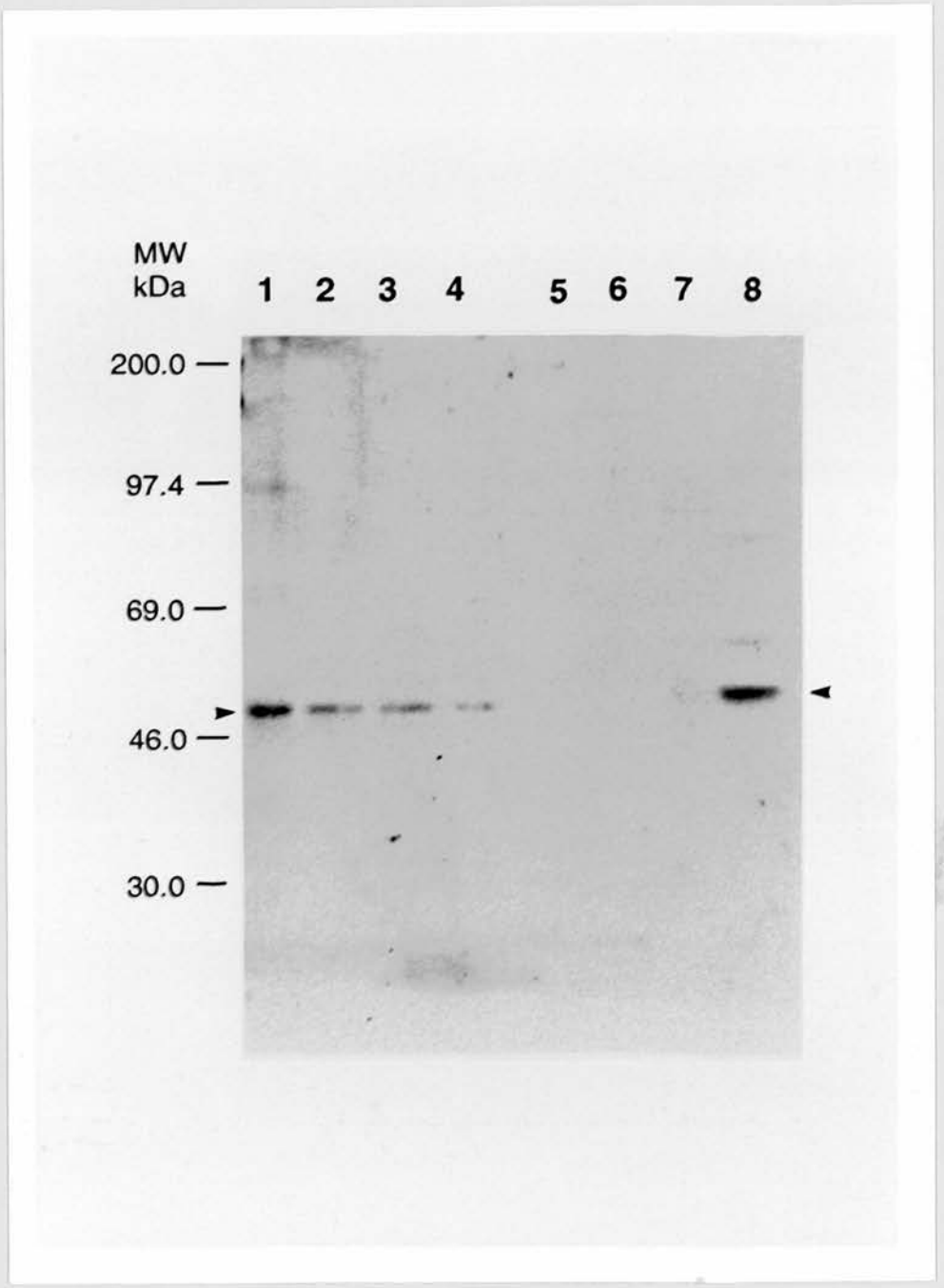

Figure 7.7 Zoo blot of SDS-solubilized mammalian sperm protein samples subjected to SDS PAGE and probed with the anti-sp55^{sox} IgG. The blot is a representative example. Lanes 1-4 contain solubilized human sperm protein from 4 different donors; lane 5 contains solubilized guinea pig sperm; lane 6 solubilized hamster sperm; lane 7 solubilized mouse sperm and lane 8, solubilized rat sperm. As before, the anti-sp55^{sox} IgG detected a 55kDa protein in human spermatozoa, and it also detected a major band, migrating at around 58kDa, in rat spermatozoa. The antibody failed to cross-react with spermatozoa from the other species. All lanes contained the equivalent of 5 x 10^6 spermatozoa and the positions of the molecular weight markers are indicated to the left of the panel.

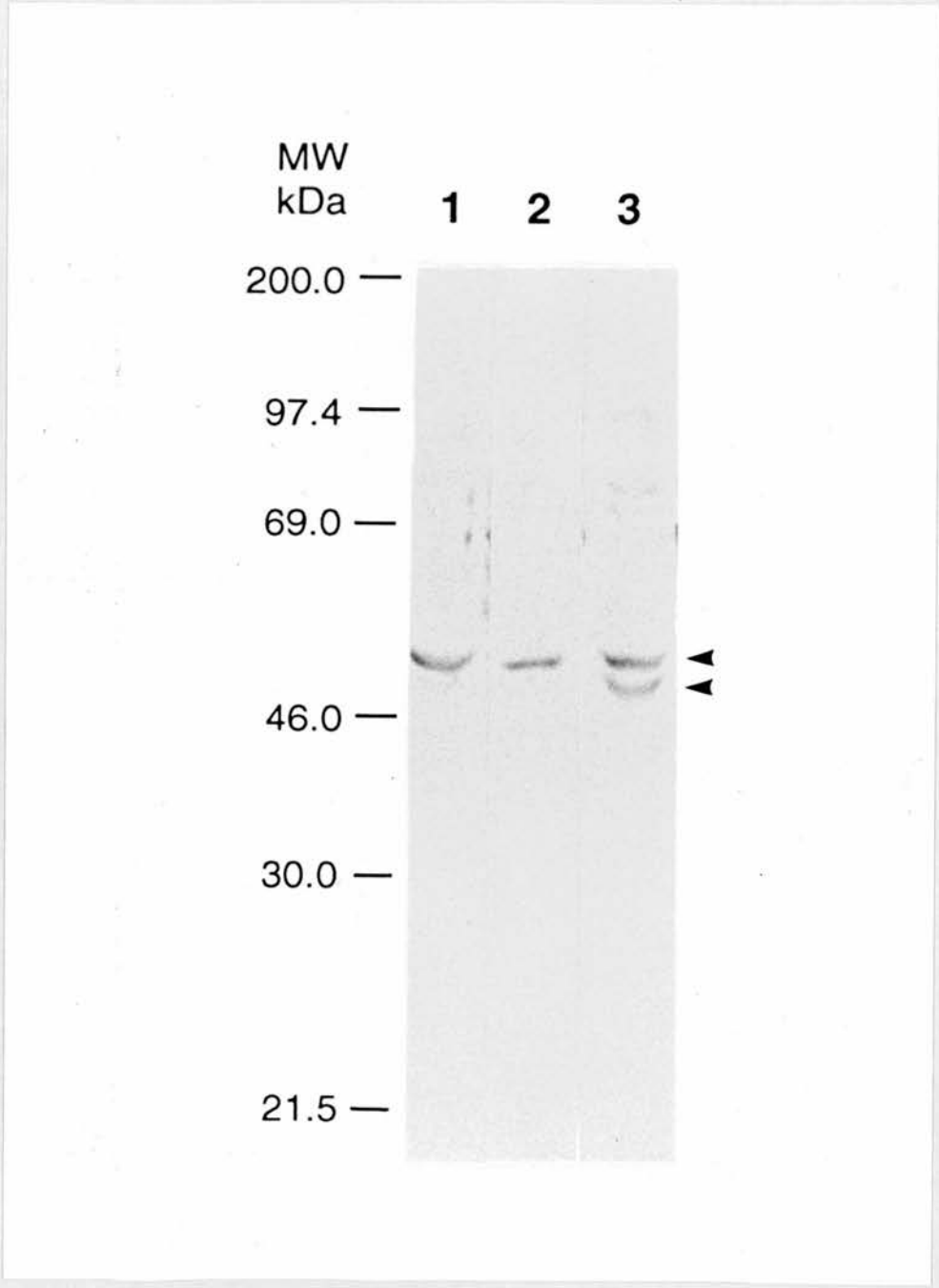

Figure 7.8 Western blot showing changes in the expression of antigen detected by the anti-sp55^{sox} IgG during the epididymal maturation of rat spermatozoa. This is a representative example. SDS-solubilized rat sperm protein was subjected to SDS PAGE and then probed with the anti-sp55^{sox} IgG. Lane 1 contains solubilized sperm from the cauda epididymis; lane 2, solubilized sperm from the corpus epididymis; and lane 3, solubilized sperm from the caput epididymis. The anti-sp55^{sox} IgG detected a band migrating at around 58kDa in caudal, corpus, and caput spermatozoa, and also detected an additional band, migrating at around 55kDa, in caput spermatozoa (see arrows). All lanes contained the equivalent of 5 x 10^6 spermatozoa and the positions of the molecular weight markers are shown to the left of the panel.

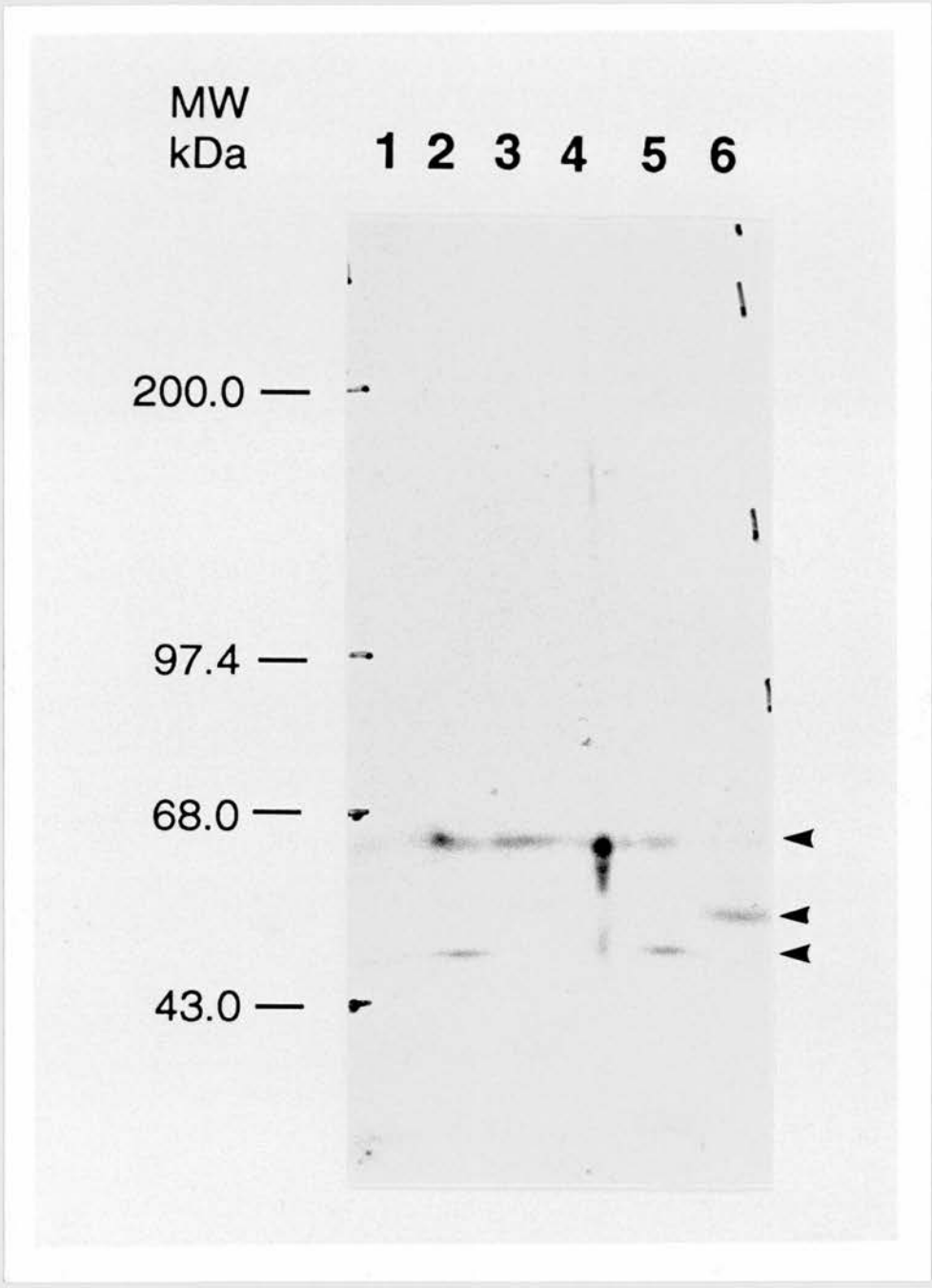

Figure 7.9 Human multiple tissue Western blot showing the tissue-wide expression of antigen recognized by anti-sp55^{sox} IgG. A commercially prepared human multiple tissue blot was probed with the anti-sp55^{sox} IgG. All samples were SDS-solubilized homogenates. Lane 1 contains brain; lane 2, heart; lane 3, kidney; lane 4, lung; lane 5, skeletal tissue; and lane 6, liver. The antibody detected a 50kDa protein, present in brain, heart, and skeletal tissue; a 55kDa protein, present in liver and kidney only; and a 65kDa protein that was present, at various levels, in all the tissues examined here. Each lane contained 75µg of protein and the positions of the molecular weight markers are shown to the left of the panel. For further details on the blot see text.

human and rat.

Human spermatozoa

Indirect, immunofluorescent staining of human spermatozoa with the anti-sp55^{sox} IgG, stained the entire surface of the spermatozoon, with the acrosome, mid-piece, and distal tail region being particularly heavily stained (Figures 7.10 and 7.11). No real differences existed between the staining patterns observed with the spermatozoa of different donors. Similarly no difference existed between the staining patterns of 100% (Figure 7.10) and 50% (Figure 7.11) spermatozoa, although some of the 50% spermatozoa did have cytoplasmic droplets that stained intensely, and the mid-pieces of the 50% spermatozoa were generally larger than the mid-pieces present in the 100% spermatozoa and again, these stained up very intensely. In some instances the acrosomal region of the spermatozoon was not stained, perhaps indicative of acrosome reacted spermatozoa.

Rat spermatozoa

A similar staining pattern was observed in the rat, with the acrosome and distal tail region being the most intensely stained components of the cells (Figures 7.12 and 7.13). The overall staining of the caudal spermatozoa (Figure 7.13) was not very intense, whilst the spermatozoa of the caput epididymis showed much greater fluorescent staining (figure 7.12).

7.3.5 Effect of anti-sp55^{sox} IgG on NADPH-induced superoxide anion generation by human spermatozoa.

Figure 7.14 illustrates the effect of anti-sp55^{sox} IgG and CRS on NADPH-induced superoxide anion generation by intact human spermatozoa and by sperm OTG extracts. In the case of the OTG extract, a slight, though non-significant, effect was observed; the anti-sp55^{sox} IgG slightly reducing the response to NADPH. The reverse was true for intact spermatozoa. In this

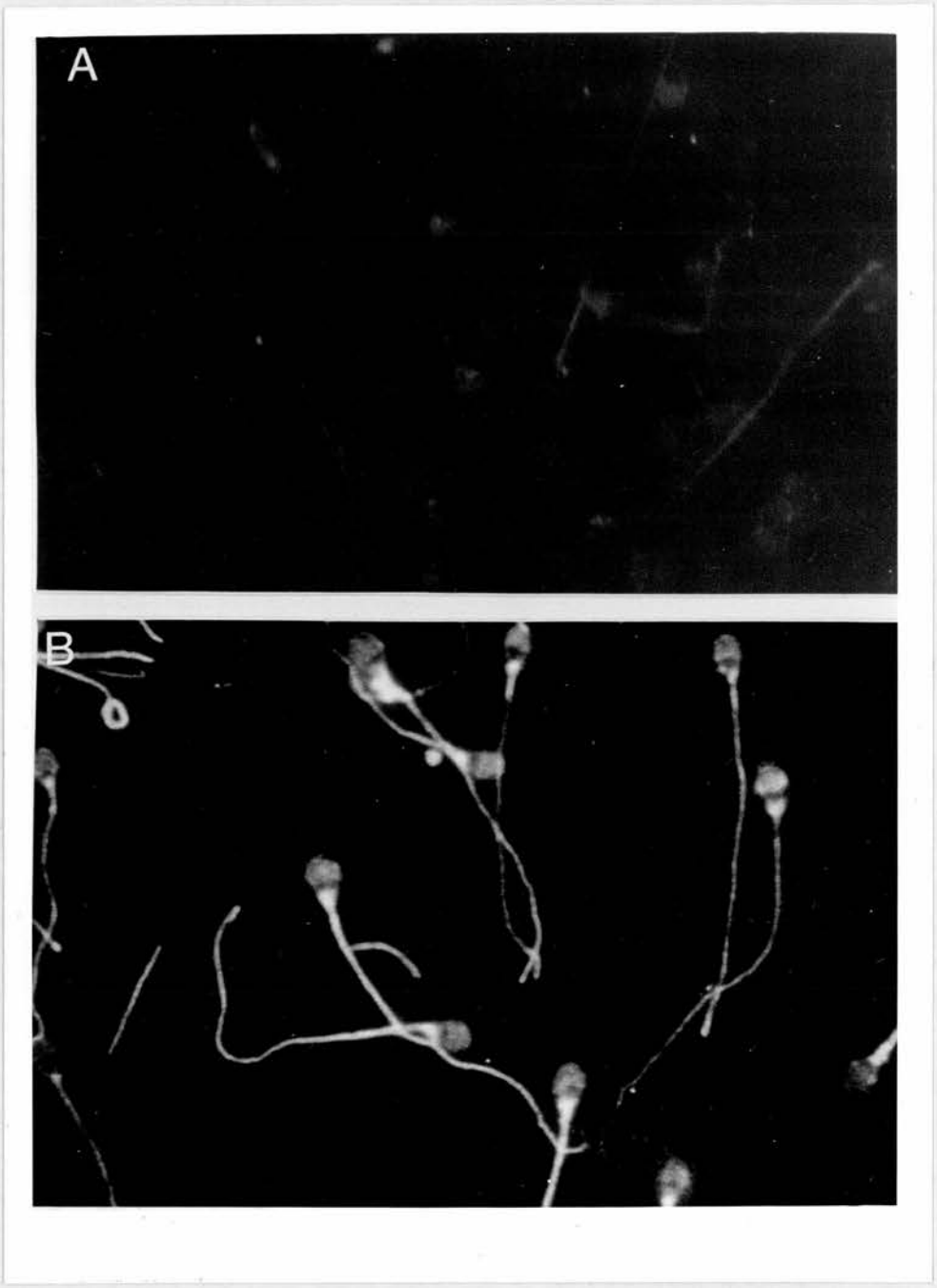

Figure 7.10 Representative indirect immunofluorescent staining of human spermatozoa from the 100% Percoll fraction. Panel A: control immunofluorescence, pre-immune rabbit serum was used in place of the primary antibody. Panel B: Anti-sp55^{sox} IgG-dependent immunofluorescence.

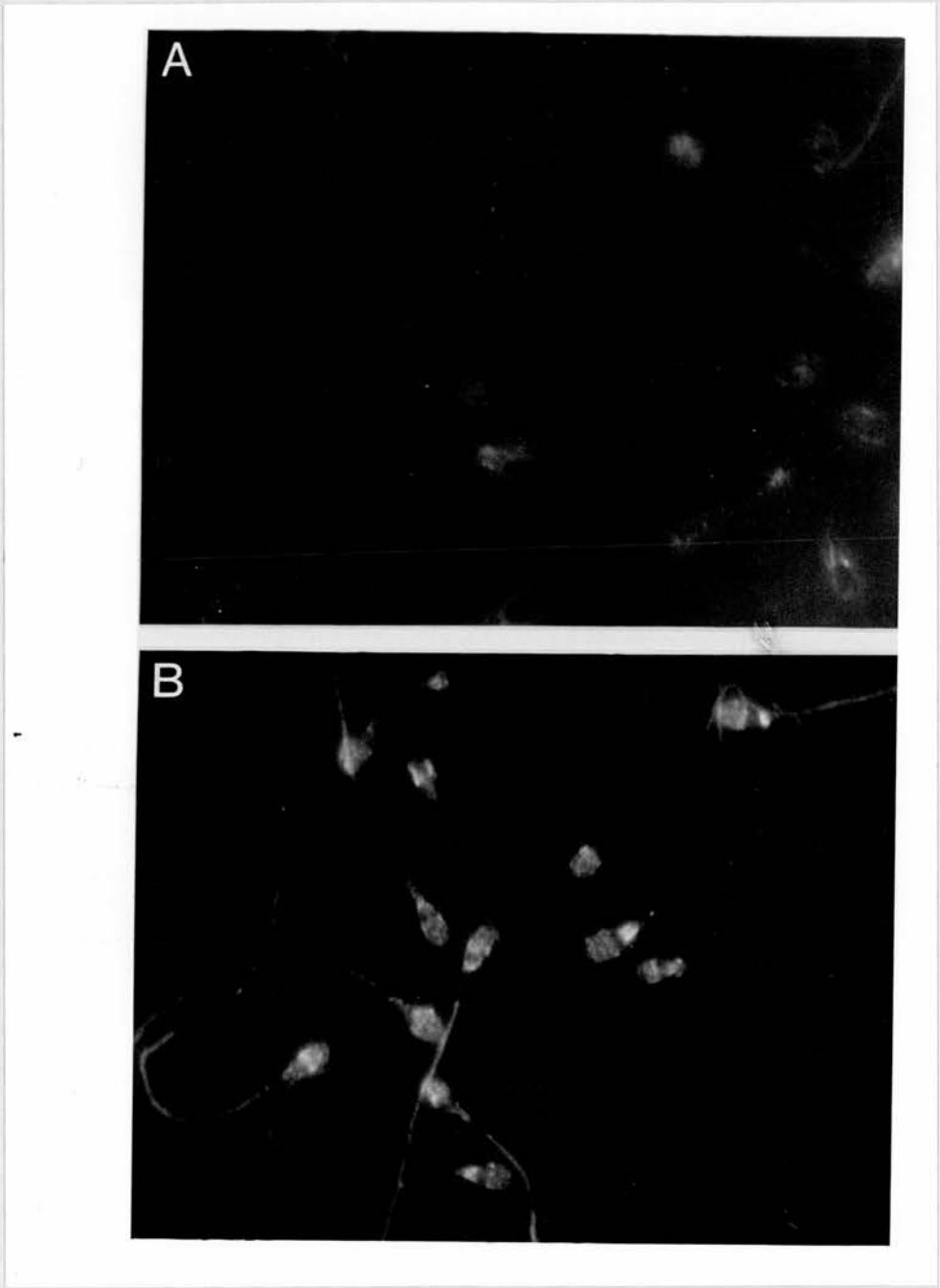

Figure 7.11 Representative indirect immunofluorescent staining of human spermatozoa from the 50% Percoll fraction. Panel A: control immunofluorescence, pre-immune rabbit serum was used in place of the primary antibody. Panel B: Anti-sp55^{sox} IgG-dependent immunofluorescence.

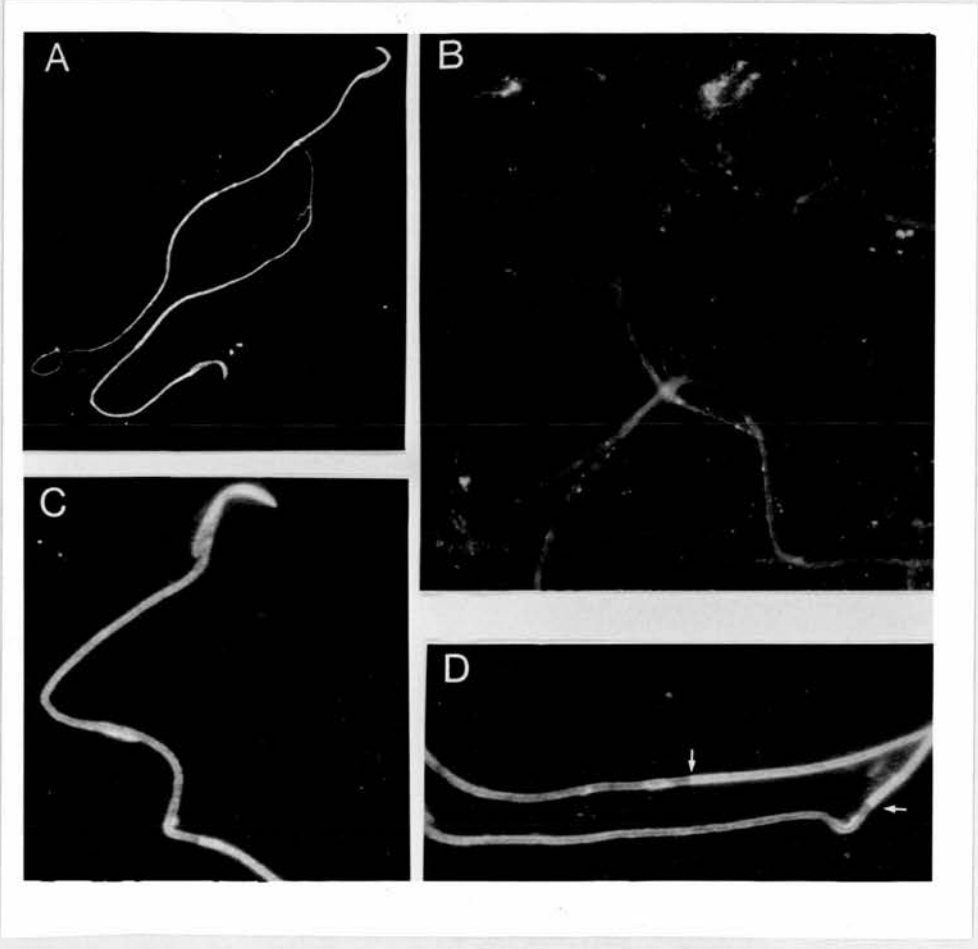

Figure 7.12 Representative indirect immunofluorescent staining of rat spermatozoa from the caput epididymis. Panels A, D and C: Anti-sp55^{sox} IgG-dependent immunofluorescence. Panel B: control immunofluorescence, pre-immune rabbit serum was used in place of the primary antibody.

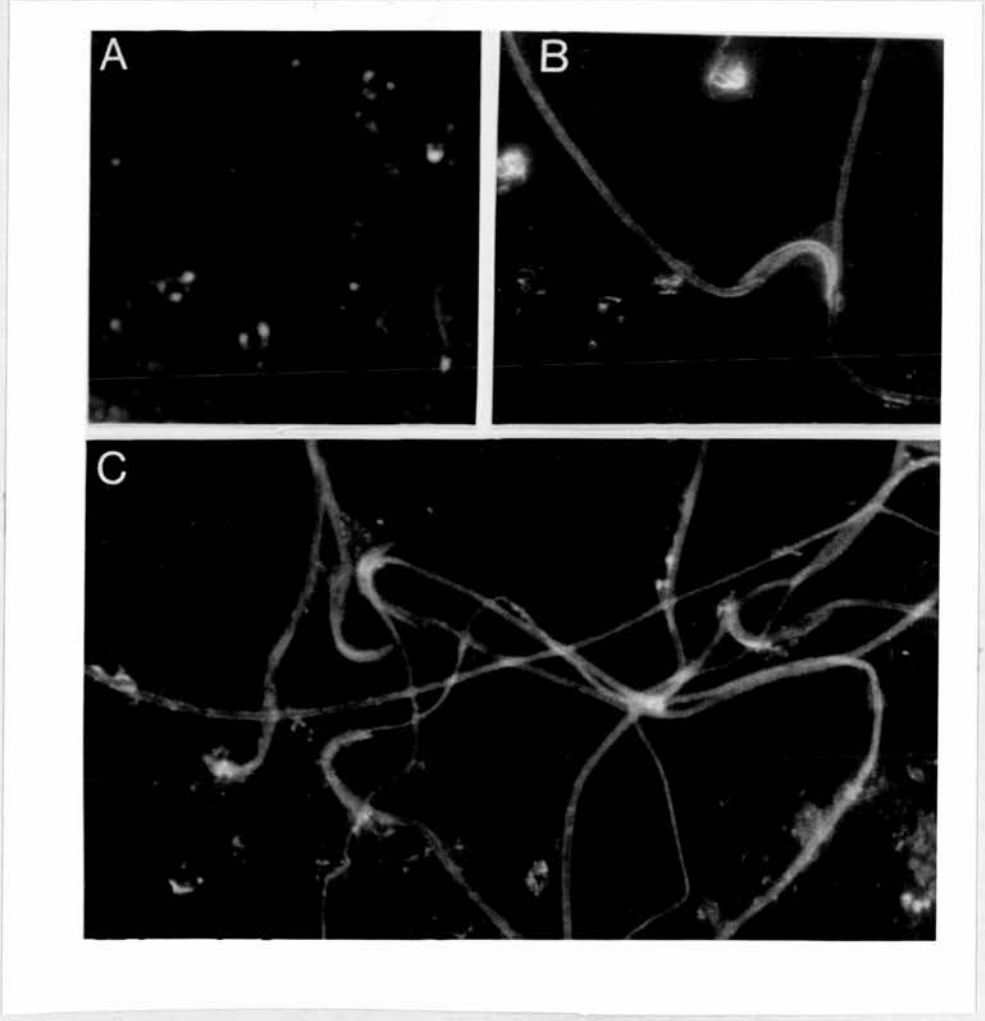

Figure 7.13 Representative indirect immunofluorescent staining of rat spermatozoa from the cauda epididymis. Panel A: control immunofluorescence, pre-immune rabbit serum was used in place of the primary antibody. Panels B and C: Anti-sp55^{sox} IgG-dependent immunofluoresecence.

instance the anti-sp55^{sox} IgG caused an increase in the response to NADPH, though again, the increase was not statistically significant, and was mirrored, to some extent, by the NADPH-response from cells incubated with CRS. Overall, no statistically significant effect of the anti-sp55^{sox} IgG on NADPH-induced superoxide anion generation by the intact spermatozoa or the OTG extract, was observed.

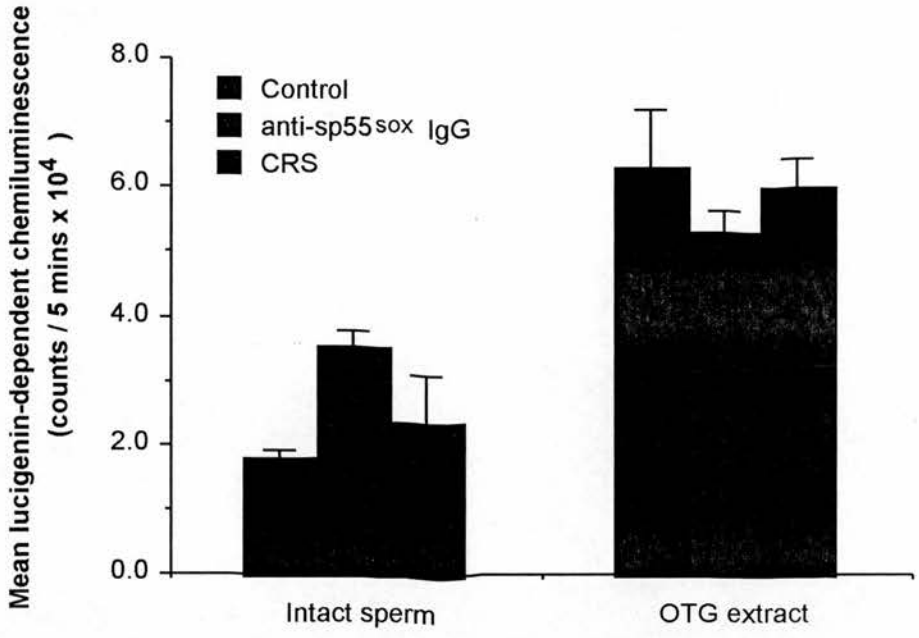

Figure 7.14 Effect of anti-sp55^{sox} IgG on NADPH-induced superoxide anion generation by intact and OTG solubilized, human spermatozoa. Prior to stimulation with NADPH (500μM), cell suspensions and extracts, except controls, were treated with anti-sp55^{sox} IgG or CRS (both at a dilution of 1/25). Subsequent lucigenin-dependent chemiluminescence was monitored. The means ±S.E. of 3 separate experiments are shown. The anti-sp55^{sox} IgG did not have any significant effect on the response to NADPH.

7.4 Discussion

The previous chapters of this thesis have lead to the identification of a 55kDa protein which is probably a component of the superoxide generating system of human spermatozoa. The goal of the work carried out in this chapter was to purify enough of the sp55^{sox} antigen to allow the raising of a polyclonal antibody, with which to study further the biochemical properties, expression

and identity of the sp55^{sox} antigen.

Although the antigen purification process was successful, resulting in sufficient protein to provoke an immune response, it did show some variation in its efficiency, i.e. the total yield of antigen obtained. This was possibly due to one or both, of two reasons. Firstly, the affinity column matrix may have become exhausted due to persistent use, reducing its protein-binding efficiency, and hence reducing the amount of protein specifically bound to the matrix. Secondly, populations of human spermatozoa are inherently heterogeneous and thus differences in the protein content of the different populations, or in the affinity of the protein for the affinity matrix, may have varied quite dramatically. The Western blot analyses of human sperm proteins with the anti-sp55^{sox} IgG illustrates this point, showing that the spermatozoa from individual donors did indeed show variation in the level of expression of the sp55^{sox} antigen. Other studies have shown that there is great variation and heterogeneity in the protein composition of the spermatozoa between, and within sperm populations (Mallidis *et al.*, 1991).

The antigen, at the quantities used, did provoke an immune response from the host animal, resulting in the production of polyclonal antibodies specifically recognising the protein of interest, i.e. the 55kDa protein. However, the immune-response did not appear to increase on repeated exposure to the antigen, as expected, the antibody response to the final boost being no greater than the response to the initial boost. This may have been due to the comparatively low quantity of antigen used for the final boost, or due to a rapid clearance of the newly injected antigen, again resulting in much lower levels of circulating antigen. In this context, an insufficient time interval between boosts may have been a factor since high levels of antigen-specific antibodies may have been in circulation at the time of the second booster injection (Harlow and Lane, 1988). Alternatively, some sort of de-sensitization or tolerance process may have occurred.

Relatively high levels of IgG were purified from the anti-sp55^{sox} polyclonal anti-serum. This was to be expected, as the secondary immune response, evoked by repeated immunization with the same antigen, generally leads to an enhanced clonal selection process for the IgG secreting B lymphocytes (Roitt *et al,* 1985). This occurs due to surface bound IgG antibody receptors having greater affinity for the available antigen than the receptors of B lymphocytes secreting other antibody classes. Thus, B cells expressing and secreting IgG type antibodies will be preferentially stimulated to mature and proliferate at the expense of B cells expressing and secreting other classes of immunoglobulin, e.g. IgM, the predominant antibody class of the primary immune response (Roit *et al,* 1985). This phenomenon of preferential class proliferation is known as the 'class shift', and it is a typical result of hyperimmunization, i.e. repeated immunization with the same antigen (Harlow and Lane, 1988).

The IgG immunoglobulin fraction was purified from the crude anti-serum by exploiting the properties of Protein G. Protein G is a 30-35 kDa protein isolated from the wall of ß-haemolytic streptococci of the C or G strains (Kronvall, 1973), and it has binding sites that interact with the Fc domain of IgG molecules, and thus allows their separation from other proteins and antibody classes. Figure 7.3 shows a typical elution profile from a Protein G affinity column demonstrating that the absorbance peak, representing the IgG antibody fraction, is in a composite form, composed of at least 2 individual peaks. This is probably due to the differing affinities of the different IgG sub-classes (IgG_1, IgG_2, IgG_3, etc.) for the protein G, thus causing them to elute differentially (Harlow and Lane, 1988).

The IgG fraction of the anti-serum was used to demonstrate that the antibody targeted the antigen it was raised against, and also to determine whether it recognised any other proteins present in spermatozoa or other tissues. It was important to determine this as the possibility existed that one or

more of the antigenic determinants (epitopes) recognised by the anti-sp55^{sox} IgG, could be present on other proteins present in human spermatozoa, or other tissues. In human spermatozoa, the antibody did recognise a protein other than the 55kDa protein. This additional protein migrated at approximately 135kDa, and was recognised only in some of the sperm protein extracts subjected to Western blot analyses. It is interesting that 135kDa is the sum of the sizes of the two dominant proteins in the affinity 2', 5' ADP fractions, i.e. 55kDa and 80kDa, and it is possible that these two proteins exist as a fairly tightly associated complex that is not always completely dissociated upon SDS treatment. Thus, epitopes present on the isolated 55kDa protein, may also be exposed on the putative, 135kDa complex. However, more stringent denaturation protocols involving reducing conditions failed to eradicate the 135kDa band, perhaps indicating that more severe denaturing conditions still, were required to fully dissociate the protein complex, or that the 135kDa protein was an individual sperm protein component, not in any way associated with the 55kDa protein, but sharing some of the same epitopes. It is also possible that the 135kDa protein is a precursor protein, that is post-translationally processed to produce the 55kDa protein, sp55^{sox}, and the 80kDa protein. The majority of the evidence available does seem to point to the 135kDa protein being a complex, containing the 55kDa protein and the 80kDa protein, but to fully address this point an antibody would have to be raised against the 80kDa protein from the affinity chromatography, and this antibody used to probe the same blot as the anti-sp55^{sox} IgG. Such experiments with the two antibodies would determine whether or not they cross reacted with the same 135kDa band, and show whether the two proteins did form a 135kDa complex. It would also be very useful to try other, much more stringent extraction protocols, in an attempt to fully dissociate any protein complexes present in human spermatozoa. It does seem peculiar that the 135kDa protein is only detected on some blots. If the sub-units of a complex are very tightly

associated, it is doubtful that in some instances they would be very easily dissociated, without harsh, denaturing conditions. However, as has already been pointed out, human sperm suspensions are very heterogeneous in their composition, and this fact may be enough to result in the observed discrepancies between the cross-reactivities of the spermatozoa from different individuals.

It would be, perhaps, interesting and possibly very revealing, to determine whether or not the pattern of staining was conserved, over time, in different sperm samples from the same individual. If it could be shown that the appearance of the 135kDa band was not merely a result of slightly different protein extraction conditions, then the staining pattern could possibly be used as a marker of sperm quality, and could possibly be correlated with the fertilizing ability of the spermatozoa. This type of correlation has certainly been shown with other markers of sperm maturity, e.g. LDH C_4 (Casano *et al.*, 1990), diaphorase (Gavella and Lipovac, 1992), creatine kinase (Huszar *et al.*, 1990). Another possibility arising from the inconsistent appearance of the 135Kda sperm component is that the presence and immunoreactivity of the 135kDa protein may be indicative of the general maturity of the spermatozoa in the individual samples. It is possible that post-testicular maturational events, such as those that occur in the epididymis, may result in either the loss or gain of the immunoreactivity of this band, or in the association or dissociation of its sub-units (Jones, 1989). Yet another factor to consider is that the spermatozoa from individual donors show marked variation in their ability to generate the superoxide anion, in response to exogenous NADPH (see Chapter 4). It is possible that the ability to generate the superoxide anion is somehow linked to the presence of this 135kDa band. However, to address any of the above points much work would have to be undertaken, and this work would possibly, be greatly assisted if the actual identity of the $sp55^{sox}$ protein could be determined.

It is interesting to note that 135kDa is also the size of the constitutively expressed, endothelial nitric oxide synthase (Garvey *et al.*, 1994), as mentioned in the previous chapter of this thesis. Hence, the cross-reactivity of the anti-sp55^{sox} IgG with the 135kDa protein may provide further evidence in support of the existence of NOS in human spermatozoa. It is possible that NOS is present in human spermatozoa, in the form of the 135kDa band detected here, but that sp55^{sox} is not a component of it. It may be that both NOS and the sp55^{sox} protein share similar structural motifs, resulting in shared epitopes. Many proteins and enzymes that have related functions and share similar properties, show quite substantial homology and share common sequence motifs. Examples of this phenomenon are proteins belonging to the flavoprotein, cytochrome containing, P-450 family (Porter, 1991). This family of proteins shows substantial sequence homology, which, in is manifested by, for example, very similar flavoprotein moieties. It is thought that the flavoprotein components of enzymes arose very early in the evolutionary time scale and due to this are highly conserved. The fact that the ROS generating system of human spermatozoa appears to contain a flavoprotein (Chapter 5 of this thesis) could explain the postulated cross-reactivity with NOS, an enzyme also possessing a flavoprotein component (Tracey et al., 1994, Zembowicz et al., 1993). In addition to the possibility of shared flavoprotein moieties, the fact that both enzyme systems must also possess NADPH binding sites, supports the possibility of shared epitopes between these two molecules. A very similar situation exists with the NADPH oxidase of phagocytic leucocytes, i.e. it has been shown to share similar sequence motifs with P-450 enzymes, with NOS (Segal *et al.*, 1992), and with other enzymes which possess NADPH and FAD binding domains (Rotrosen *et al.*, 1992). In fact, it has now been established that a family of flavoprotein containing enzymes exists, all of which share important sequence motifs with one another. This family is called the FNR (ferredoxin-NADP^{+} reductase) family and it includes many P-450 enzymes,

e.g. NOS, cytochrome P-450 reductase, and also the NADPH oxidase (Porter, 1991; Correll et al., 1993). Thus, it is not inconceivable that a polyclonal antibody raised against a component of an enzyme system, with putative NADPH and FAD binding sites, would show cross reactivity with molecules with similar properties and functional domains. In fact, such results are often very misleading, conveying information leading to the false belief that two molecules are one and the same, when in fact they just share a few common epitopes. Such a situation arose when antibodies raised against the NADPH oxidase of phagocytic leucocytes were shown to cross-react with glomerular mesangial cells (Neale et al., 1991; Meier et al., 1991) and fibroblasts (Radeke et al., 1991), the molecules recognised were subsequently shown not to be identical to those of the NADPH oxidase of leucocytes (Meier et al., 1993; Emmendorffer et al., 1993).

On Western blots composed of sperm protein extracts from the 50% and 100% Percoll fractions of semen samples, differences were observed in the patterns of reactivity with the anti-sp55^{sox} IgG. Protein extracts from the 50% Percoll fractions showed a greater intensity of cross-reactivity with the anti-sp55^{sox} IgG than the extracts from the 100% Percoll fraction. This was not unexpected as this fraction of spermatozoa is characterized by the presence of abnormal spermatozoa and immature germ cells (Aitken and Clarkson, 1988; Aitken *et al.*, 1994b). Numerous studies have shown that these types of cells commonly contain greater levels of enzymes, e.g. creatine kinase (Huszar *et al.*, 1990), G6PDH (Aitken et al., 1994b). The apparently increased expression of sp55^{sox} by the cells from the 50% Percoll fraction could be one explanation for the generally elevated levels of ROS generation by the cells of the 50% Percoll fraction (Aitken and Clarkson, 1988; Aitken and West, 1990; Aitken *et al.*, 1992; Aitken *et al.*, 1994b). The increased prevalence of the 135kDa band in cells from the 50% Percoll fraction could also be a plausible explanation for this .

Inclusion of the reducing agent, mercaptoethanol, in protein

solubilization buffers leads to the disruption of the disulphide bonds that are present in some proteins. Disruption of intramolecular disulphide bonds causes the native, conformational structure of the protein to be lost, i.e. the protein is denatured. Addition of mercaptoethanol to proteins can also lead to the dissociation of protein complexes due to the disruption of intercellular disulphide bonds. Thus, electrophorectically fractionating proteins that have been solubilized in the presence of mercaptoethanol, any sub-unit structure should, hopefully, be revealed and the individual components identified. Also, the migration of solubilized proteins that are not fully denatured, and which have retained some of their native structure, may be hindered.. Non-linear, protein molecules, retaining some of their native structure, pass less easily through the pores in polyacrylamide gels, and their migration is retarded. However, in the presence of mercaptoethanol the proteins are more likely to be completely denatured and have a linear structure: thus their migration through the polyacrylamide gels will not be retarded and their relative mobility will more accurately reflect the true molecular weights of the proteins.

Addition of mercaptoethanol to solubilized human sperm protein extracts did not result in obvious disruption to any subunit structure of $sp55^{sox}$ or of the 135kDa protein band, nor did it alter mobility of the two proteins. However, conducting the Western bolt analyses under reducing conditions did result in the enhancement of the detection of $sp55^{sox}$ and the 135kDa protein. It is possible that the reducing conditions revealed sperm epitopes on the two protein molecules which were reactive with the anti-$sp55^{sox}$ IgG solution, but that were not revealed under non-reducing conditions. It is noteworthy that reducing conditions not only resulted in an overall enhancement of antigen detection, but also lead to the appearance of the 135kDa band in protein samples, in which it was not detected when the analyses were conducted under non-reducing conditions. However, this was not true for every sample lacking the 135kDa protein under non-reducing conditions; in some samples

the 135kDa protein was never observed, regardless of their disulphide bond status. This raises the possibility, in some samples at least, that the disulphide bond status of human spermatoza somehow regulates the expression or immunoreactivity of the 135kDa protein. Sulphydryl status is a profound modulator of sperm function and is widely implicated in the events associated with epididymal maturation (Jones, 1989, Amann, 1993). Thus, as was suggested earlier, it may well be that the presence/absence of the 135kDa protein may well be a marker of sperm maturation.

Experiments conducted with zoo-blots, constructed from human, mouse, hamster, guinea pig and rat spermatozoa, showed that the anti-sp55^{sox} IgG only recognised epitopes present on the proteins of rat spermatozoa. It is quite surprising that the IgG only recognised antigen in the rat, and not the other species, as sperm antigens commonly show widespread, interspecies expression (Isahakia and Alexander, 1984). The main protein recognised in this instance, in rat spermatozoa, was a protein that migrated at around 58kDa. Although a few minor protein bands were picked up, no protein at 135kDa was observed, possibly indicating that the 55kDa protein is not a component of the 135kDa protein.

Subsequent Western blot analyses of rat spermatozoa at various stages of epididymal maturation, revealed that the expression of antigens recognised by the anti-sp55^{sox} IgG changed during epididymal maturation. The level of expression of the 58kDa protein did not appear to significantly change, but epididymally immature spermatozoa, i.e. those from the caput region of the epididymis, expressed an additional protein, at approximately 55kDa that also cross-reacted with the anti-sp55^{sox} IgG. The appearance of this extra protein in epididymally immature sperm is probably indicative of the maturation-associated changes that occur in the sperm plasma membrane during the cells' transit through the epididymis (Jones, 1989). Such alterations are explained more fully in Chapter 9 of this thesis, but briefly, during their transit through

the epididymis, spermatozoa undergo profound changes, resulting in quite dramatic changes in the composition of their plasma membrane and other structures, e.g. the acrosome (Phillips *et al*, 1991). It is possible that the 55kDa protein recognised in the caput spermatozoa either becomes modified so that it no longer contains epitopes recognised by the anti-sp55^{sox} IgG, or that the 55kDa protein is completely lost from the spermatozoon, during passage through the epididymis. The 55kDa protein may be very important in the epididymal maturation of the rat spermatozoon and it would be interesting to study its expression and its possible role in epididymal maturation , further. A survey of the literature on the epididymal maturation of rat spermatozoa, did not reveal any reference to a 55kDa protein that becomes modified during epididymal transit (review, Cooper, 1990). The lack of any reference to a 55kDa protein being lost during epididymal maturation perhaps indicates that the 55kDa protein is not discarded by the spermatozoon, but is somehow modified, eradicating the epitopes recognised by the anti-sp55^{sox} IgG, unless the protein is a quantitatively minor protein, that is not readily detected on silver stained SDS-PAGE gels of rat sperm proteins.

The results from the human multiple tissue Western blot show that sp55^{sox} is probably not a sperm-specific antigen, as a 55kDa protein was detected in liver and kidney. Proteins on the multiple tissue blot with other molecular weights, i.e. 60kDa and 65kDa, also cross-reacted with the anti-sp55^{sox} IgG. It is not uncommon for sperm antigens to be present in somatic tissue and for them to share similar epitopes with non-identical, somatic counterparts (Chaffee and Schachner, 1978), although some sperm antigens are gamete specific and this property has allowed them to be investigated for contraceptive vaccine purposes (Primakoff, 1994). For a contraceptive strategy to be employed, based upon the development of a sperm antigen-based vaccine, the immunogenic intervention must only interfere with processes specifically involved in fertilization. Therefore, the molecules used must be

gamete specific, so that no generalized effects on other, non-reproductive, cells and tissues occur (Jones, 1994). Thus even if the sp55^{sox} antigen was shown to have a profound role in fertilization, due to its apparent lack of gamete-specificity, it could not be a candidate for contraceptive vaccine development. The 135kDa protein was not detected in the multiple tissue blot, perhaps indicating that the 135kDa protein in human spermatozoa is a sperm-specific antigen.

The immunofluorescent staining of human spermatozoa with the anti-sp55^{sox} IgG did not show particularly startling results. The sp55^{sox} and/or 135kDa protein was shown to be widely distributed over the entire surface of the spermatozoon, showing that the immunogenic determinants of the antigen(s) exist throughout the plasma membrane. However, certain regions did stain more intensely than others, indicative of the continuous, but differentiated nature of the domains on the sperm plasma membrane (Friend, 1982; Olsen and Winfrey, 1991). The surface of the spermatozoon is very heterogeneous, with extensive diversity in the arrangement of intramembraneous molecules, and this differential arrangement of molecules results in the identification of at least 5 principal, discrete surface domains. The principal domains are those overlying the anterior acrosome, equatorial segment, post-acrosomal region of the head, midpiece, and principal piece of the flagellum, and it is thought that these domains are established fairly early on during spermiogenesis (O'Brien and Millette, 1984). Using probes such as lectins and antibodies the localization of molecules to specific domains has been widely reported (Koehler, 1975; Feuchter *et al.*, 1981; Myles *et al.*, 1981; Olsen and Winfrey, 1991-review), demonstrating that the sperm plasma membrane is a highly differentiated structure. The heterogeneous nature of the sperm plasma membrane probably reflects the diversity of the functions that its various regions have to perform during fertilization (Jones *et al.*, 1983).

The domains preferentially stained with the anti-sp55^{sox} IgG were the

acrosomal region, midpiece, and principal tail segment. It is interesting that the mid-piece of the spermatozoon was heavily stained as this is the location in which many cytoplasmic enzymes are retained by the spermatozoon, e.g. G6PDH (Angelov and Dokov, 1977), and the area where some enzymes become incorporated into the plasma membrane, e.g. LDH C_4 (Wang *et al.*, 1990). It is possibly also significant that the tail piece should stain intensely, as it has been shown that a possible physiological role of the superoxide anion in human spermatozoa is in the control of hyperactivated movement (de Lamirande and Gagnon, 1993a and b). It can be appreciated that if the short lived superoxide anion is to be involved in the regulation of motility, then its genesis must occur in a location close to where it can exert an effect, i.e. on the flagellum and on enzymes controlling motility.

Even though post-testicular spermatozoa have little or no ability for lipid or protein biosynthesis, they are equipped to modify and degrade existing molecules (Bedford and Hoskins, 1990) and hence, the characteristics and composition of the plasma membrane domains may change during epididymal maturation of the spermatozoon (Jones, 1989). Immunofluorescent staining of rat caput and cauda epididymal spermatozoa did not reveal any alteration in the distribution of the targeted antigen(s) during epididymal maturation, but did reveal changes in the intensity of the fluorescent staining. When viewed alongside the results obtained from the Western blot analyses of rat epididymal sperm, this was to be expected. The spermatozoa from the caput epididymis showed much brighter fluorescence than the spermatozoa from the cauda epididymis, and this is possibly due to the existence of the additional immunoreactive band in the caput spermatozoa. The reduced fluorescence of the cauda spermatozoa could also be indicative of a masking of the immunoreactive antigens during epididymal maturation.

Finally, what evidence is there supporting the direct involvement of $sp55^{sox}$ in superoxide anion generation by human spermatozoa? The anti-

sp55^{sox} IgG did cross-react with one of the bands on non-denaturing PAGE gels, shown to be responsible for superoxide anion generation, as indicated by NBT staining. This band is, generally, the second most active in terms of superoxide anion generation, and was postulated, in Chapter 6, to be possibly LDH C_4. This now can be ruled out as LDH is composed of four 35kDa subunits, not 55kDa sub-units. However, although the anti-sp55^{sox} IgG did cross-react with one of the bands responsible for superoxide anion generation, it did not succeed in significantly inhibiting NADPH-induced superoxide anion generation by human spermatozoa. Antibodies raised against the NADPH oxidase of phagocytic leucocytes do inhibit ROS generation by this complex (Yea *et al.*, 1990). The conflicting results obtained in this study could be due to a the fact that only quite low concentrations of the antibody could be used. Concentrations of the anti-sp55^{sox} IgG higher than 1/25, resulted in spurious luminometer signals, so the antibody may have not been present at a high enough concentration to bind to the superoxide generating system at levels great enough to inhibit its activity. Another possibility is that the anti-sp55^{sox} IgG does not recognise epitopes involved in either NADPH-binding or electron transfer.

7.5 Summary and conclusions

A polyclonal antibody was raised against the 55kDa protein, designated sp55^{sox} and the subsequently purified IgG fraction of the antiserum was used to determine the proteins distribution, tissue and species-specificity, and its role in NADPH-induced superoxide anion generation by human spermatozoa.

The anti-sp55^{sox} IgG consistently recognised a 55kDa protein in human spermatozoa, and also recognised a 135kDa protein in some instances. The anti-sp55^{sox} IgG did not specifically recognise antigens present only in human spermatozoa, but recognised a variety of proteins in other human tissues. The anti-sp55^{sox} IgG also detected a 58kDa antigen in rat spermatozoa which was

present throughout epididymal maturation, and which was, in the caput region, accompanied by an additional cross reactive protein with a molecular mass of 55kDa. Immunofluorescent labelling studies carried out with the anti-sp55^{sox} IgG, showed that antigen recognised by the IgG was located over the entire surface human spermatozoa, although the antigen appeared to be concentrated over the acrosomal, mid-piece and principal tail regions of the spermatozoon. The anti-sp55^{sox} IgG also fluorescently labelled rat spermatozoa in a similar fashion, and showed a marked decline as the spermatozoon underwent epididymal maturation.

The anti-sp55^{sox} IgG did specifically label one of the protein bands in human spermatozoa capable of superoxide anion generation, but did not significantly inhibit or enhance superoxide anion generation by intact human spermatozoa, or sperm OTG extracts.

In conclusion, the sp55^{sox} protein is an antigenic component of human spermatozoa which appears to be a component of the one of the superoxide generating systems of these cells, and this system is possibly a protein complex, involving sp55^{sox} and an 80kDa protein. The anti-sp55^{sox} IgG appears, in human spermatozoa, to specifically recognise the individual sp55^{sox} antigen and the 135kDa complex, and could be used in a protein identification strategy, i.e. immunological screening of a human testes cDNA expression library.

Chapter 8

Molecular characterization and identification of $sp55^{sox}$.

8.1 Introduction

A protein, apparently involved in ROS generation by human spermatozoa, designated $sp55^{sox}$, has been isolated from human spermatozoa and used to generate a polyclonal antibody. Up until now the protein has not been identified, but the possibility exists that it is a component of an already known enzyme, e.g. nitric oxide synthase. In order to resolve the true nature of this protein, recombinant DNA technology was utilized.

The realization that deoxyribonucleic acid (DNA) was the carrier of the genetic information of heredity by Avery and colleagues (Avery *et al,* 1944) and the subsequent discovery of the double helix structure of DNA by Watson and Crick (Watson and Crick, 1953), led to rapid progress in the deciphering of the genetic code. In was quickly realized that DNA sequences dictated the amino acid sequence of the corresponding peptide, and that a, non-overlapping, triplet code of nucleic acids was responsible. Evidence for this was first provided by Crick *et al* (1961) and then confirmed by other workers in the same year (Nirenberg and Matthaei, 1961; Lengyel *et al,* 1961).

Progress in molecular biology, particularly recombinant DNA technology, has been extremely rapid in recent years, and has lead to the development of numerous, powerful, techniques which have revolutionized virtually all fields of biology. The most important of these techniques, with regard to the work described in this thesis, is the development of DNA cloning, the subsequent production of cDNA libraries, methods to screen these libraries using nucleic acid and immunological probes, and finally techniques enabling the rapid sequencing of DNA and prediction of amino acid sequences.

Molecular techniques have developed very rapidly, and are now in forms enabling the relatively rapid and simple identification, cloning and sequencing of the DNA's encoding their corresponding proteins. In fact, recombinant DNA technology has lead to the demise of the more conventional techniques of protein purification and direct amino acid sequencing of proteins, since it is often much easier, more rapid and efficient to deduce the amino acid sequence of a particular protein by the molecular cloning route, than by the more traditional biochemical methods.

Many DNA cloning strategies involve the insertion of stretches of foreign DNA into bacteriophage vectors, especially bacteriophage λ, which has the very useful feature in that the central third of its genome is not necessary for lytic growth. This property of the bacteriophage λ was first discovered by Campbell in 1971, and it was revealed that a region of the bacteriophage genome, that lying between the J and N genes, could be replaced by a variety of *E. coli* DNA segments. This property of bacteriophage λ, has led to it occupying a central role in molecular cloning, and since its first use in 1974 (Murray and Murray, 1974; Rambach and Tiolais, 1974; Thomas *et al,* 1974) it has been extensively developed to create versatile and sophisticated vectors. The points in the bacteriophage genome where foreign DNA can be inserted are the sites at which restriction nucleases act. A restriction nuclease is a bacterial enzyme that protects it from viral attack by degrading the DNA molecules carried into the cell by viruses (Nathan and Smith, 1975). Restriction nucleases recognise specific stretches of DNA, and cleave the DNA at these points. One such 'restriction enzyme' is *Eco* R1 (Smith, 1979). Restriction enzymes cut double-helical DNA into DNA fragments, some of which, such as *Eco* R1, result in DNA fragments with short, single stranded tails at the two ends of each fragment. These single-stranded ends are called 'cohesive ends', as each tail can form complementary base pairs with the tail of any other end produced by the same restriction enzyme. The formation of

these 'cohesive ends' allows any two DNA fragments to be easily joined together, as long as the fragments were generated with the same restriction enzyme. In this way, a fragment of DNA can be inserted into the genome of a self replicating genetic element such as a bacteriophage or plasmid, such that the latter becomes a vector for the insertion of foreign DNA into a bacterial host, which will act as the factory for the production of the foreign DNA (Alberts *et al,* 1989). This is DNA cloning. Some bacteriophage λ vectors have only a single target for the insertion of foreign DNA, and these are known as insertion vectors, DNA only being added not lost, whilst some bacteriophage λ vectors have a pair of sites, flanking nonessential, bacteriophage DNA, which is replaced by the foreign DNA. These vectors are known as 'replacement ' vectors, i.e. some phage DNA is lost and replaced with foreign DNA (Sambrook *et al,* 1989).

A cDNA library can be created by purifying the mRNA of interest, which is then used as a template for reverse transcriptase, an enzyme produced by retroviruses, which synthesizes complementary DNA by copying RNA. This DNA is known as cDNA and it can then be converted from single stranded, to double stranded DNA by DNA polymerase. The resulting double stranded cDNA molecules can then be inserted into a bacteriophage or plasmid vector and cloned in a host cell. The entire collection of clones derived from one mRNA preparation constitutes a cDNA library. cDNA libraries, because they are made from mRNA, are composed of DNA sequences encoding only proteins expressed in the particular cells, tissues, or organ from which the original mRNA was purified (Alberts *et al,* 1989).

A commonly used bacteriophage insertion vector for cDNA library production, is λgt 11 (Huynh *et al,* 1985). The genome of this vector carries a portion of the *E. coli* ß-galactosidase gene, including the upstream elements essential for its expression. Within the carboxy-terminal coding region of this gene, is a single Eco R1 site, into which foreign DNA can be inserted. In

appropriate bacterial hosts, expression of the chimeric gene can be induced, resulting in the synthesis of a fusion protein consisting of the amino-terminal portion of the ß-galactosidase gene fused to the down stream open reading frame of the foreign DNA. The expression of the chimeric gene is induced using isopropyl-1-thio-ß-D-galactoside (IPTG). If an intact ß-galactosidase gene is expressed, i.e. non-chimeric, blue plaques will appear if the phage have been plated in the presence of the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal). Chimeric gene expression produces clear plaques and hence, recombinant phage can be easily distinguished from their non-recombinant counter-parts (Sambrook *et al,* 1989). Thus cDNA libraries constructed in λgt 11 can be screened immunologically for the expression of specific antigens in *E.coli* (Young and Davis, 1983), and by using a testis-specific cDNA library, less screening will probably have to be performed before the relevant clone is identified, as the library will be enriched in cDNA molecules encoding testis-specific proteins. A second advantage of using a cDNA library is that the clones will contain the uninterrupted, coding sequence of a gene.

As mentioned, a cDNA library constructed in λgt 11 is an expression library and can be screened with antibodies, resulting in the identification of clones expressing fusion proteins containing epitopes recognised by the antibody. The cDNA library can be screened with polyclonal or monoclonal antibodies. Monoclonal antibodies have the advantage of being very specific, and thus little non-specific background binding is observed during screening. However, they have the disadvantage in that they only recognise one epitope, and therefore the number of recombinant clones they will detect will be reduced and many plates may have to be screened before a positive clone is identified. Polyclonal antibodies, which normally recognise many epitopes on a protein have an increased chance of recognising and cross-reacting with a clone that expresses a fragment of the protein of interest. However, polyclonal

anti-sera often contain antibodies that will cross-react with non-recombinant protein components of the bacterial lysate, and thus may result in false positives or high background. Fortunately, such non-specific, cross-reacting proteins can be removed from an anti-serum before screening is commenced, and therefore, this property of a polyclonal antibody does not have to remain a significant problem (Obar and Holzbaur, 1993).

This chapter describes the immunological screening protocol devised for the identification of testis-specific cDNA clones encoding the sperm protein $sp55^{sox}$, and the subsequent, partial nucleic acid, automated sequencing of a positive clone.

8.2 Materials and Methods

8.2.1 Absorption of the primary antibody with *E.coli* lysate.

Polyclonal antibody preparations often contain significant quantities of antibodies that cross-react with *E. coli* proteins, and these contaminating antibodies will increase background binding, and possibly result in the identification of false positives, thereby affecting the sensitivity and reliability of the assay. Such antibodies can be removed, and the associated problems eliminated, by following an antibody absorption protocol, such as the one described below.

Preparation of E. coli lysate

A single colony of streaked out *E. coli* Y 1090 from an LB plate (20 LB agar capsules, from Bio 101 Inc., La Jolla, CA, USA; in 500ml distilled water) containing 50µg/ml ampicillin (Sigma), was grown up overnight in 100ml LB broth (13 LB broth capsules from Bio 101 Inc.; in 500ml of distilled water) containing 0.2% maltose (Sigma), at 37°C, with good aeration, in a Gallenkamp orbital incubator.

The cells from the overnight culture were harvested at 5000*g* for 10 minutes at 4°C. The supernatant was removed and the cells were resuspended in 3ml of 50mM Tris-HCl (Sigma), pH 8.0; 10mM EDTA (Sigma), pH 8.0. The suspension was freeze-thawed 4 times, and then sonicated at full power for 6 periods of 20 seconds each, using a Soniprep Ultrasonicator (MSE). The suspension was cooled on ice between bursts to minimise any heating effects. The resulting cell extract was then centrifuged at 12,000g for 10 minutes at 4°C, the supernatant decanted, and stored at -20°C until use.

Absorption of anti-sp55^{sox} IgG with the E. coli lysate

The IgG solution was diluted 1:10 with TBS containing 5% milk powder. 0.5ml of *E. coli* lysate was used per ml of IgG solution (before dilution), and the lysate/antibody mixture was incubated for 4 hours at room temperature, on a rotating wheel, to ensure good mixing. The mixture was then stored at 4°C overnight and then spun at 13,000rpm to remove any precipitated *E.coli* protein-antibody complexes. The resulting absorbed antibody solution was stored at 4°C, in the presence of 0.05% sodium azide (Sigma), until it was used for immunological screening.

Testing of Absorbed antibody against a dot blot of E. coli lysate

Any remaining cross-reactivity of the pre-absorbed IgG solution was assessed by preparing a dot blot of sperm protein and the *E. coli* lysate. A 3 by 3 dot blot was constructed, composed of a human sperm suspension (4, 3, and 2µl of 20 x 10^6/ml); *E. coli* lysate (1µl of neat, 1/10, and 1/100 lysate), or SDS-solubilized sperm (4, 3, and 2µl of an extract composed of 5 x 10^6/25µl SDS extraction buffer). The blot was allowed to dry and then subjected to the usual ECL detection system, using the absorbed primary antibody at a dilution of 1:200, and the secondary antibody (anti-rabbit IgG, HRP-conjugated, from

Amersham) at a dilution of 1:6000. The usual protocol was followed and the resulting blot developed with the ECL detection system from Amersham.

8.2.2 Library screening

A commercially available human testicular cDNA library, cloned into the expression vector, lambda gt11 (λgt11; Clontech, Cambridge, UK) was screened using the pre-absorbed, anti-sp55^{sox} IgG.

A single colony of *E. coli* Y 1090, streaked out on an LB agar plate containing 50μg/ml ampicillin (Sigma), was grown up overnight in 20ml LB broth, containing 0.2% maltose and 10mM Mg SO_4, at 37°C, with good aeration, in a shaking incubator. For each 245mm x 245mm plate, 4ml of the Y 1090 overnight culture, was mixed with 125μl of lambda diluent (10mM Tris-HCl, pH 7.5; 10mM $MgCl_2$ (BDH), 0.1mM EDTA) containing 4.5 x 10^5 plaque forming units (pfu)/ml. The phage/Y 1090 culture was incubated for 15 minutes at 37°C to allow phage attachment. The infected cells were then plated onto LB plates using 50ml of LB top agar (13 LB broth capsules and 3.6g of Bacto Agar from DIFCO, Detroit, MI, USA; in 500ml of distilled water) containing 40μg/ml X-gal (Gibco). The plates were allowed to set for 10 minutes, and then incubated, upside down, at 42°C, for approximately 4 hours, or until small plaques were just visible. The plates were then overlaid with dry nitrocellulose filters (Hybond C-super, Amersham) which had previously been saturated with 10mM IPTG (Gibco, Paisley, Scotland, UK), the substance which induces the expression of the ß-galactosidase gene. The plates were then incubated for 3.5 hours at 37°C, after which time, the nitrocellulose filter was removed, placed in TBS containing 0.1% Tween-20 (Sigma), and replaced with a second filter. The plates were incubated for a further 4 hours at 37°C, after which time the second filter was removed.

The filters were then blocked with TBS containing 5% milk powder for one hour and subjected to the usual ECL immunological detection protocol, as

follows. The primary antibody was the pre-absorbed anti-sp55^{sox} at a dilution of 1:200 in TBS containing 5% milk powder and 0.1% Tween-20. The filters were incubated in the primary antibody solution overnight at 4°C. The filters were subjected to the usual washing steps, and then incubated for 1 hour in the secondary antibody solution, which was anti-rabbit IgG conjugated to HRP (Amersham) at a dilution of 1:6000. The filters were washed again and then positive plaques were identified using the ECL Western blotting detection system from Amersham.

Any positive plaques were removed from the plates in a bore of agar taken from the plate with the wide end of a Pasteur pipette. The agar plugs were placed in a 1.5ml, sterile Eppendorf tube, and the phage eluted with 1ml SM buffer (20mM Tris-HCl, pH 7.4; 100mM NaCl, 10mM Mg SO_4) overnight. After phage elution, a drop of chloroform (Sigma) was placed in each tube to kill the plating *E. coli*. The phage stocks were then stored at 4°C until use.

For secondary screening the above procedure was repeated, with the following modifications. 150mm plates were used and hence, the quantities of everything was scaled down. For each 150mm plate, 600µl of a Y 1090 overnight culture was mixed with 100µl of phage stock (from the primary screening) in lambda diluent containing 2.5 x 10^4 pfu/ml. The phage was allowed to absorb as before, and was then plated onto an LB plate using 7.5ml of top agar, containing 40µg/ml X-gal. The plates were incubated as before, and again duplicate lifts taken, which were subjected to the detection protocol outlined above. Positive plaques were isolated as above and a tertiary round of screening undertaken, identical to the secondary screening, with the phage stock from the secondary screening. After tertiary screening, single, well isolated, positive plaques were retrieved, eluted, and then subjected to further analysis.

8.2.3 Preparation of phage lysates

In order to purify phage DNA, containing the insert encoding antigens recognised by the ant-sp55^{sox} IgG, a phage lysate was prepared from a liquid culture of the positively identified phage. Briefly, 200µl of phage eluent, was added to 250µl of a fresh overnight culture of Y 1090. The mixture was incubated at 37°C for 20 minutes and then used to inoculate 50ml of LB broth containing 10mM Mg SO_4. The culture was incubated in the shaker at 27°C until lysis occurred, usually after around 7 hours. At this point, 125µl of chloroform was added and the culture shaken for a further 15 minutes at 37°C. The culture was spun at 8000*g* for 10 minutes to pellet the cellular debris, the supernatant removed, transferred to a sterile container, and stored at 4°C until use. for polymerase chain reaction (PCR) analysis.

8.2.4 Polymerase chain reaction

The DNA insert of the positive phage was amplified using the polymerase chain reaction (PCR) technique (Saiki *et al,* 1985). PCR was performed using a PCR reagent kit containing Taq DNA polymerase, 10 X buffer, and Mg Cl_2; Perfect Match™ , both from Promega (Promega Corporation, Southampton, UK), and deoxynucleoside triphosphate (dNTP) polymerization mix, containing dATP, dCTP, dGTP and dTTP, from Pharmacia. The pair of primers used were synthesised, in-house, using an oligonucleotide synthesizer (Applied Biosystems, Warrington, Cheshire, UK) and were based upon the sequence data of the λgt 11, up (forward primer) and downstream (reverse primer) of the *Eco* R1 site. The forward primer (F1) was 22 bases in length and was located 16 to 37 base pairs (bp) upstream of the *Eco* R1 site, and had the sequence 5'-GGTGGCGACGACTCCTGGAGCC-3'. The reverse primer (R1) also consisted of 22 bases and was located 23 to 44 bp downstream of the *Eco* R1 site and had the sequence 5'-GACACCAGACCAACTGGTAATG-3'. The reaction mixture consisted of a total volume of 100µl and contained the

following; Taq DNA polymerase, 2.5 Units; dNTP's, each 200µM; 1 X reaction buffer (10mM Tris-HCl, pH 9.0; 50mM KCl, 0.1% Triton X-100); Mg Cl_2, 1.5mM; perfect match, 1µl (1U); forward primer (F1), 0.5µM; reverse primer (R1), 0.5µM; 70µl DNA template. DNA from the phage lysate preparation was used as the DNA template. Immediately prior to use, 5µl of lysate + 65µl water were mixed and heated to 70°C for 5 minutes, and then kept on ice until they were added to the other components of the PCR mix.

The PCR reaction was performed in a Techne PH C-3 programmable heating block (Techne (Cambridge) Ltd., Cambridge, UK). The PCR had an initial melt temperature of 94°C for 5 minutes and was then followed by 30 cycles of annealing (2 minutes at 50°C), extension (2 minutes at 72°C) and melting (2 minutes at 94°C). The final extension was carried out at 72°C for 7 minutes. The PCR products were then analysed by agarose electrophoresis and then stored at 4°C until further use.

8.2.5 Agarose gel electrophoresis

Agarose gels were run using a Hoefer Minnie™, submarine agarose gel unit, model HE33 (Hoefer Scientific Instruments, Newcastle, Staffs., UK). 8% agarose gels were prepared with Sea Kem® GTG® agarose (FMC Bioproducts, High Wycombe, Bucks. UK) in 0.5 X TBE (Tris, 45mM; boric acid, 45mM; EDTA, 1mM; pH 8.0) and containing 0.6µl/ml ethidium bromide (Sigma). All samples were mixed with 1µl of tracking dye (0.25% Bromophenol blue, 0.25% xylene cyanol FF, from Sigma; 15% Ficoll, from Pharmacia; all in water) and then applied to the gel along with the appropriate DNA markers. Gels were run at a constant voltage of 100V for 1-2 hours in 0.5% TBE. Gels were then viewed and photographed under UV illumination in order to identify the DNA fragments present.

8.2.6 Subcloning into pCR™ II

To enable simple and rapid sequencing of the phage DNA insert, the PCR fragment was sub-cloned into the plasmid vector, pCR™ II, via the TA Cloning® system, using the TA Cloning® Kit from Invitrogen® (Invitrogen Corporation, San Diego, CA, USA). The PCR product was used without any prior modification, as follows. Briefly, the ligation reaction was carried out by incubating 1µl of the neat PCR product with the following TA Cloning® kit components; 6µl sterile water; 1µl 10 x ligation buffer; 2µl pCR™ II vector; and 1µl T4 DNA ligase. The ligation reaction mixture was made up in a 500µl sterile Eppendorf tube, and incubated at 12°C overnight.

The TA Cloning® transformation was performed as follows. One vial of One Shot™ Competent Cells were thawed on ice along with a vial of 0.5M ß-mercaptoethanol. A vial of SOC medium (20mg/ml bacto-tryptone; 5mg/ml bacto-yeast extract; .5mg/ml NaCl; 2.5mM KCl; 10mM $MgCl_2$; 20mM glucose; pH 7.0), supplied with the kit,was thawed at room temperature. The overnight ligation reaction was briefly spun down, and then 1µl of it added to the thawed, vial of competent cells, to which 5µl of the ß-mercaptoethanol had already been added. The transformation mixture was mixed by very gently tapping the vial, so as not to damage the cells, and then incubated on ice for 30 minutes. The vial was then incubated in a water bath, at 42°C, for exactly 30 seconds and then placed on ice for exactly 2 minutes. 450µl of the SOC medium was then added to the vial and it was incubated in the shaker for 1 hour, at 37°C. The vial, now containing the transformed cells, was placed on ice until use.

The transformed cells were plated out on LB agar plates containing 50µg/ml ampicillin and 40µg/ml X-gal. 25µl aliquots and 100µl aliquots were spread on the plates and these were incubated, inverted, at 37°C overnight. White, positive, colonies were picked out and streaked out on LB agar plates, along with a couple of blue, negative colonies.

8.2.7 Validation of positive pCR™ II clones

Before isolation of the plasmid DNA from the positive clones, the clones were subjected to PCR analysis to ensure that they were true positives, and did contain the DNA insert of interest. Briefly, a single clone from each of the probable positive streaked out colonies, and one from a negative colony, were lyzed with 50µl of plasmid lysis buffer (20mM Tris-HCl, pH 8.4; 2mM EDTA, 1% Triton X-100). The mixture was vortexed and incubated at 95°C for 5 minutes, and then centrifuged at 13,000rpm for 5 minutes. The PCR protocol was as before, except the primers used were the Sp6 primer and the T7 primer. The pair of primers used were synthesised, in-house, as before, and were based upon the sequence data of the pCR™ II , up (forward primer) and downstream (reverse primer) of the *Eco* R1 site. The forward primer, Sp6, was a 17-mer and had the sequence 5'-ATTTAGGTGACACTATA-3'. The reverse primer, T7, consisted of 22 bases and had the sequence 5'-GTAATACGACTCACTATAGGGC-3'. The template consisted of 9µl of water and 1µl of plasmid lysate from the positive or negative clones. The actual PCR was carried out as described before, and the PCR products analyzed by agarose gel electrophoresis.

8.2.7 Isolation of plasmid DNA

Plasmid DNA was isolated from white pCR™ II clones which proved to be true positives using the Magic™ Miniprep plasmid DNA purification system from Promega. Briefly, single, isolated, insert-positive colonies were grown up overnight in 10mls of LB buffer containing 0.2% maltose, 10mM Mg SO_4, and 50µg/ml ampicillin. The cultures were incubated in the shaker, at 37°C, overnight and then spun at 3000*g* for 15 minutes to pellet the cells. The supernatant was discarded and the cells resuspended in 200µl of the Magic™ Miniprep kit re-suspension buffer. 200µl of lysis buffer (again from the Magic™ Miniprep kit) was then added to the cell preparation and it was mixed

by inversion. 200µl of neutralizing solution (from the kit) was then added to the preparation and it was spun at 14, 000 rpm for 5 minutes. The supernatant was removed and placed in a new tube. 1ml of DNA purification resin was added to the supernatant and the supernatant /resin preparation was mixed and then applied to a spin column. The column was washed with 1ml of washing buffer, and then spun at 13,000 rpm for 20 seconds. Finally 50µl of sterile water, at 65°C, was applied to the column, which was again spun for 20 seconds, and the filtrate was collected. The filtrate contained the plasmid DNA, and this was stored at 4°C, until it was ready to be used in the sequencing experiments.

8.2.8 Sequencing

Sequencing of the 1.9kb insert encoding the antigen $sp55^{sox}$ was performed using The Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (401113) from Applied Biosystems and a Applied Biosystems 373A DNA Sequencer (ABI, Warrington, Cheshire, UK).

Sequencing reactions

The sequencing reactions were performed with the purified plasmid DNA, in 500µl Eppendorf tubes. Each reaction mix consisted of 5µl purified plasmid DNA (approximately 1µg), 4µl 5 X TACS buffer (400mM Tris-HCl, 10mM $MgCl_2$, 100mM $(NH_4)_2SO_4$, pH 9.0), 1µl dNTP mix, 1µl each of the DyeDeoxy™ Terminators (i.e. ddA, ddT, ddG, and ddC), 0.5µl AmpliTaq® DNA Polymerase (4 U), 1µl primer (3.2pmols), and 4.5µl of distilled water: thus each reaction was in a total volume of 20µl. The primers used were Sp6, T7 (as described earlier), and the forward and reverse M13 primers. The Forward M13 primer was supplied with the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit, and was 18 bases long, with the following sequence, 5'-TGTAAAACGACGGCCAGT-3'. The reverse M13 primer was made in house,

was 17 bases long, with the following sequence, 5'-CAGGAAACAGCTATGAC-3'. Prior to commencement of the sequencing reactions the contents of each tube were overlaid with a drop of mineral oil.

The sequencing reaction was performed in a Perkin Elmer Cetus Model 480 thermal cycler (Perkin Elmer, Beaconsfield, Bucks, UK). Prior to placing the tubes in the thermal cycler it was pre-heated to 95°C, and then tubes were placed in the cycler. The thermal cycling of the sequencing reaction consisted of 25 cycles of the following; rapid thermal ramp to 95°C, 95°C for 30 seconds, rapid thermal ramp to 45°C, 45°C for 15 seconds, rapid thermal ramp to 60°C, 60°C for 4 minutes. At the end of cycle 25 there was a rapid thermal ramp to 4°C, which was held until the tubes were retrieved for further processing.

Precipitation of sequencing reactions

At the end of the thermal cycling of the sequencing reactions the actual reaction mix was removed from the tube and transferred to a new tube, leaving behind the mineral oil. To extract the excess terminators, 100µl of phenol:H_2O:chloroform (68:18:14, from ABI) was added to each tube. The tubes were then vortexed and centrifuged at 13,000rpm for 2 minutes. The bottom, pink layer, containing the excess terminators was then removed and a further 100µl of phenol:H_2O:chloroform added. The tubes were vortexed and spun as before. The top, clear, layer, containing the extension products, was transferred to a new tube and precipitated by adding 15µl 2M sodium acetate, pH 4.5 (BDH), and 300µl ice cold 100% ethanol (BDH). The precipitation was allowed to proceed for 1 hour at 4°C, and then the tubes were centrifuged at 13,000rpm for 30 minutes. The supernatants were removed, the pellets were washed with 100µl of ice cold 75% ethanol, and the tubes were then centrifuged at 13,000rpm for 2 minutes. The supernatants were removed and the pellets were allowed to air dry. Just before sequencing, the samples were

resuspended in 4µl loading buffer (100µl formamide (Sigma), 20µl 50mM EDTA, pH 8.0).

Automated sequencing

Automated sequencing gels were prepared as follows. In a 100ml glass beaker, 50g urea (Sigma) and 15ml 40% acrylamide (Anachem, Luton, Beds., UK) were made up to 80ml with distilled water. A spatula of the ion exchanger Amberlite MB-1 (Sigma) was added and then the mixture was dissolved by being heated gently whilst stirred. The resulting solution was filtered, 10ml of 10 X TBE added, and made up to 100ml with water. Immediately prior to pouring, the gel was polymerized with 45µl TEMED and 500µl 10% AMPS (both BioRad). The gel was allowed to set for 2 hours before use.

Prior to sample application gels were pre-run at 1600 V for 15 minutes, in 1 X TBE. Before sample application, the samples were heated to 95°C for 5 minutes, and then applied to the gel. Gels were run overnight and the data collected on an Apple Macintosh computer. The data was analyzed using the GeneJockey II© programme (Biosoft, Cambridge, UK), and final, consensus sequence data used to conduct a homology search of Genbank and EMBL sequence data banks. Homology searches were kindly conducted by Dr Philippa Saunders, utilizing the Seqnet database, located at Daresbury, Cheshire, UK.

8.2.9 Western blotting

Sequence database searches using the consensus sequence data, revealed significant sequence homology with human collagen, type V1. In order to explain this apparent homology, Western blot analyses were conducted on human sperm protein extracts, and on solubilized human collagen type VI (Calbiochem), with the anti-sp55^{sox} and with a polyclonal antibody raised against human collagen, type VI.

SDS-solubilized human sperm protein (equivalent of 5 x 10^6 cells /lane) and acid solubilized human collagen, type VI (10µg/lane), were separated on 7.5% acrylamide gels by SDS-PAGE, under reducing conditions, as previously described in this thesis. Pre-stained SeeBlue™ molecular weight markers were used, 10µl/lane.

Post-electrophoresis, SDS-PAGE gels were blotted onto Hybond C super nitrocellulose (Amersham) by the semi-dry method described earlier. Post-blotting, the nitrocellulose membranes were subjected to the usual antibody-based, antigen detection protocol, with the following primary antibodies; anti-sp55^{sox} IgG, and anti-human collagen, type VI polyclonal antibody purchased from Biomol (Biomol Fine Chemicals GmbH, Hamburg, Germany). The primary antibodies were used at dilutions of 1:200 and 1:500 respectively. Membranes were blocked, as normal in 10% milk powder in TBS, and then incubated in primary antibody overnight at 4°C. The secondary antibody used was donkey anti-rabbit IgG, HRP conjugate (Amersham), and was used at a dilution of 1 in 6000, membranes were incubated in this antibody for 1 hour at room temperature.

After incubation with the secondary antibody, the nitrocellulose membranes were subjected to the usual washing procedure and then processed for antigen detection using the enhanced chemiluminescent Western blotting detection kit from Amersham. This procedure was carried out according to the manufacturers instructions, as already outlined in the materials and methods chapter of this thesis (Chapter 3).

8.3 Results

Pre-absorption of the anti-sp55^{sox} with the *E. coli* lysate resulted in almost all cross-reactivity of the antibody solution with *E. coli* proteins being removed (figure 8.1). Only neat lysate was cross-reactive with the antibody solution, and this was quite weak. Dilutions of 1/10 and 1/100 did not cross react with

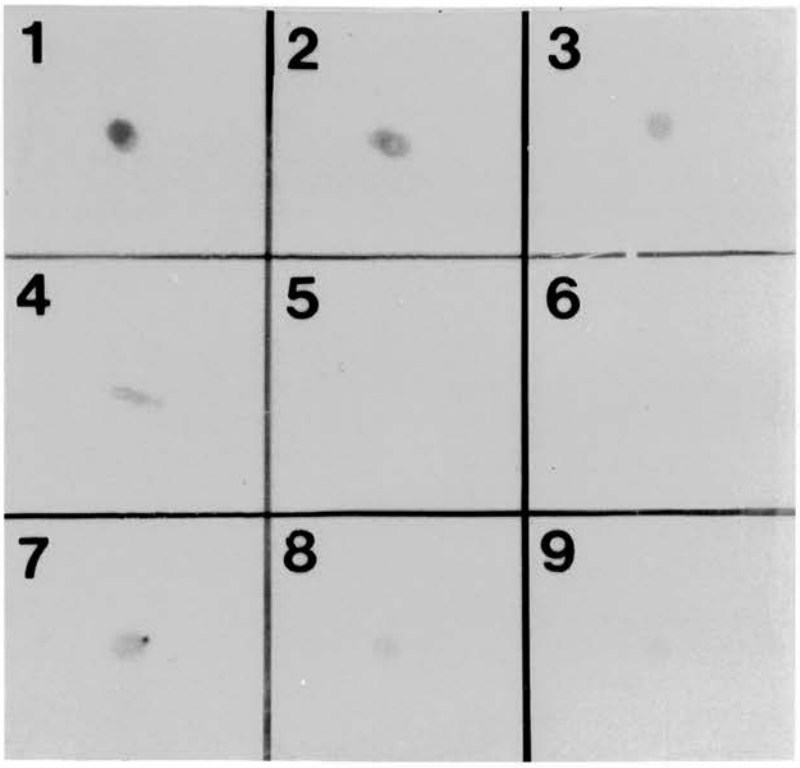

Figure 8.1 Dot blot comparing the cross-reactivity of the anti-sp55^{sox} IgG with whole human sperm, SDS-solubilized human sperm and *E. coli* lysate. 1-3, SDS-solubilized human sperm extract; 4-6, *E. coli* lysate; and 7-9, whole sperm. 1, 4, and 7 are undiluted preparations; 2, 5 and 8 are 1/10 dilutions; and 3, 6 and 9 are 1/100 dilutions The anti-sp55^{sox} IgG cross-reacted with all dilutions of the sperm preparations but only with the neat *E. coli* lysate.

the antibody at all, and thus the antibody solution could be deemed suitable for immunoscreening.

Immunoscreening of the human testicular cDNA library constructed in λgt 11 resulted in the identification of positive clones. In total, around 150,000 plaques were screened in duplicate and two putative positive clones were identified. However, after secondary screening, only one of these clones proved to a true positive. This clone, for further verification was taken through a tertiary round of screening, and at this point the clone was plaque purified. Figure 8.2 shows the identification of a single positive clone after primary screening and the numerous positive clones identified after the secondary round of screening.

A lambda lysate of the positive clone was produced and this was used to amplify the phage DNA insert via polymerase chain reaction (PCR). The sequence of these primers and their relative positions in the λgt 11 genome are depicted in figure 8.3. The PCR products from the lambda lysate were electrophoresed and this revealed that the PCR product was approximately 1.9kb in size (Figure 8.4). The PCR product was subsequently sub-cloned into the plasmid vector, pCR™ II. PCR of the plasmid insert, with the primers Sp6 and T7 (Figure 8.3), confirmed that the insert had been sub-cloned successfully, and again showed that the insert was approximately 1.9kb in length (Figure 8.5). The plasmid DNA was purified using the Magic™ Miniprep plasmid DNA purification system and this construct of the insert DNA was used for automated sequence analysis.

Partial sequence data for the DNA insert was obtained using the Sp6, T7, M13 F and M13 R primers (Figure 8.3). Time was very limited towards the end of this project and hence, it was not attempted to sequence the entire insert. However, a reasonable amount of good, consensus sequence data was obtained. In total, consensus sequence was obtained for 470 nucleotides; 212 using the Sp6/M13 R primers, and 258 using the T7/M13 F primers (Figure

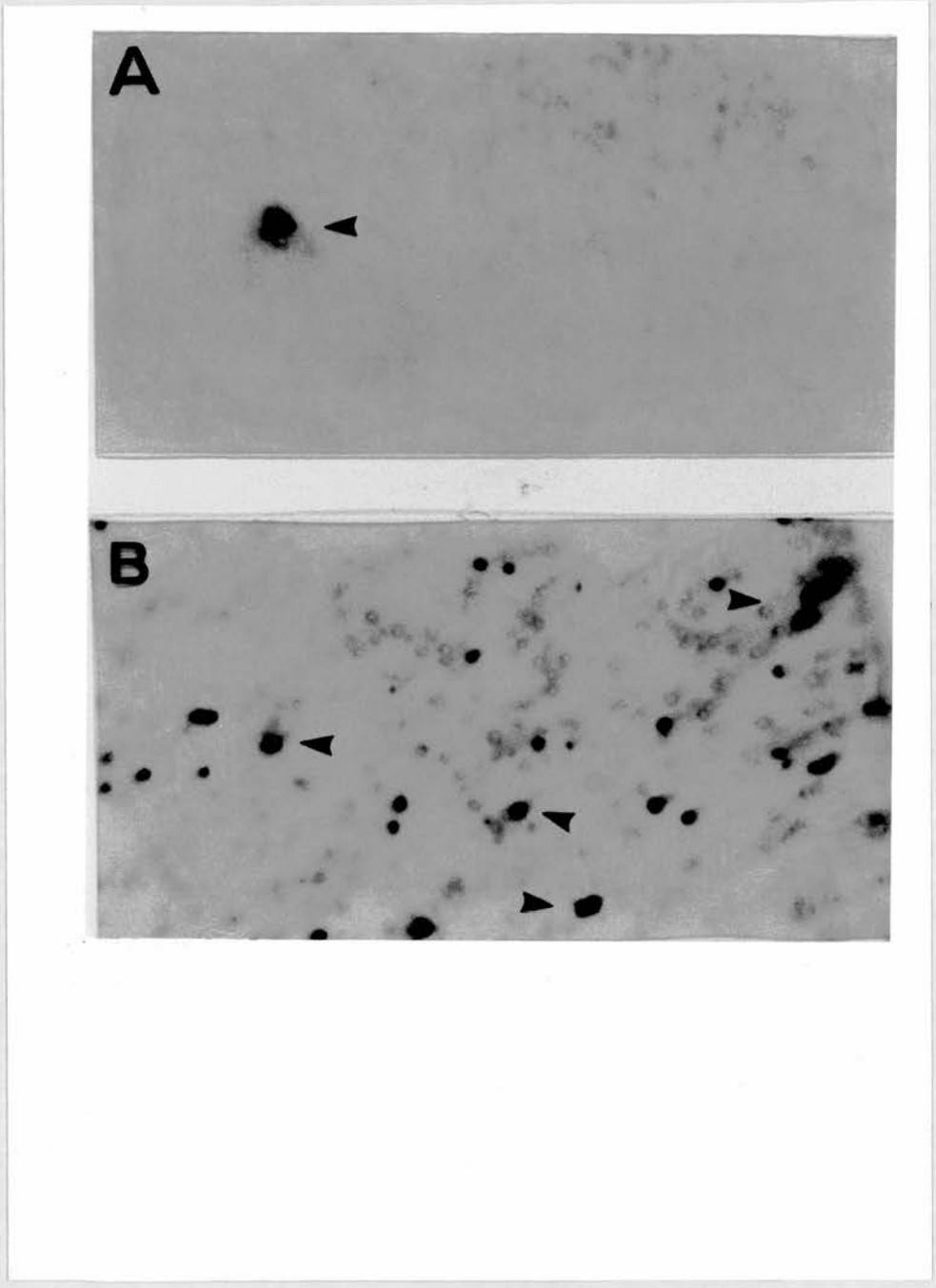

Figure 8.2 Immunoscreening of a λgt 11 testis cDNA expression library with the anti-sp55^{sox} IgG . Panel A: identification of a single positive clone after primary screening. Panel B: identification of numerous positive clones after secondary screening.

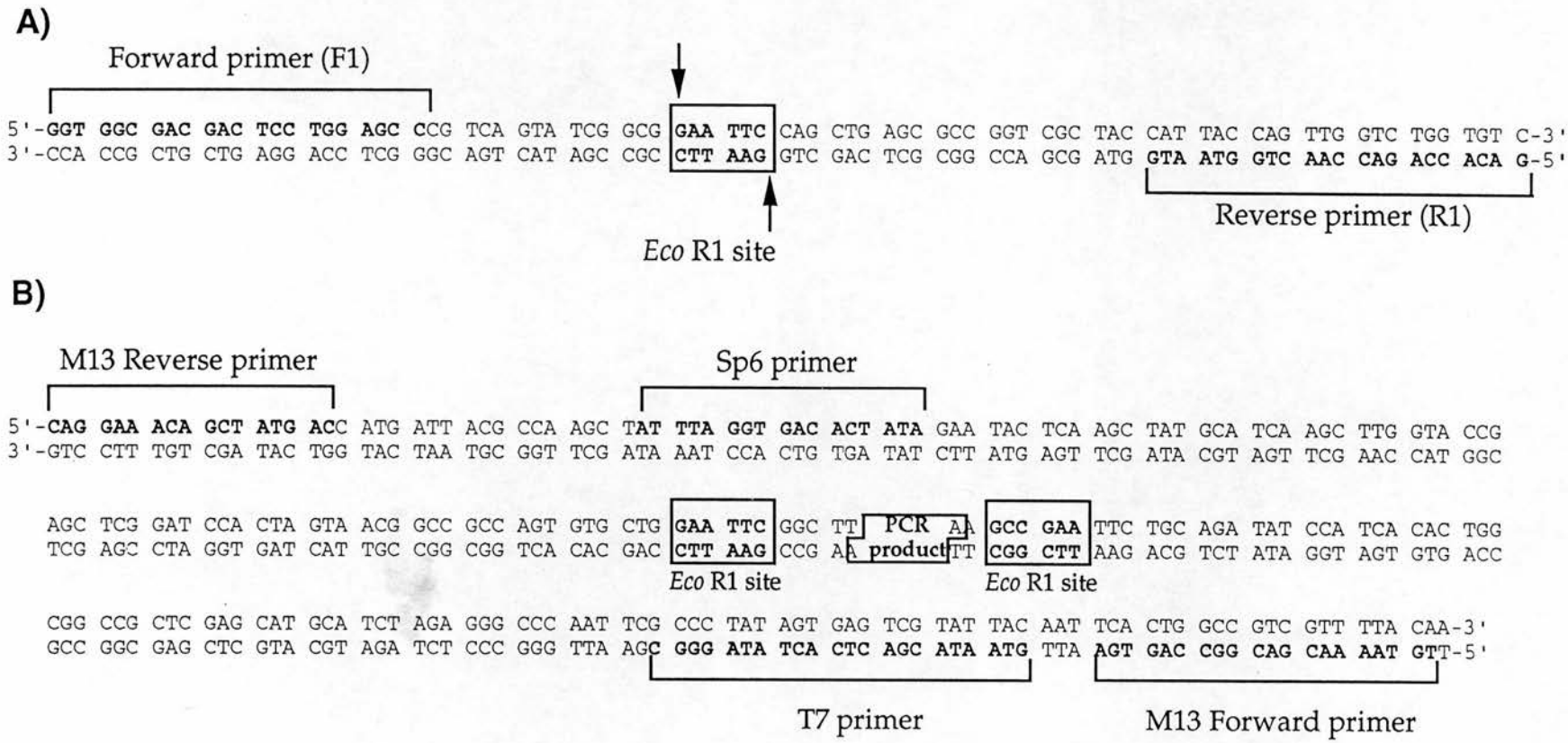

Figure 8.3 DNA sequence of A) λgt11, and B) pCR II around the *Eco* R1 restriction sites, showing the sequences and positions of the primers used during PCR amplification and sequencing of the insert DNA.

Figure 8.4 Electrophoresis of PCR products in an 0.8% agarose gel. Lane 2 contains the PCR product from the amplification of the λgt 11 positive clone; lane 2 contains the PCR product from a non-recombinant, i.e. negative λgt 11 clone; lane 3 contains the PCR product from a reaction in which no DNA template was added. The PCR product from the λgt 11 positive clone was shown to be approximately 1.9kB in size. Lanes 1 and 5 contain DNA size markers.

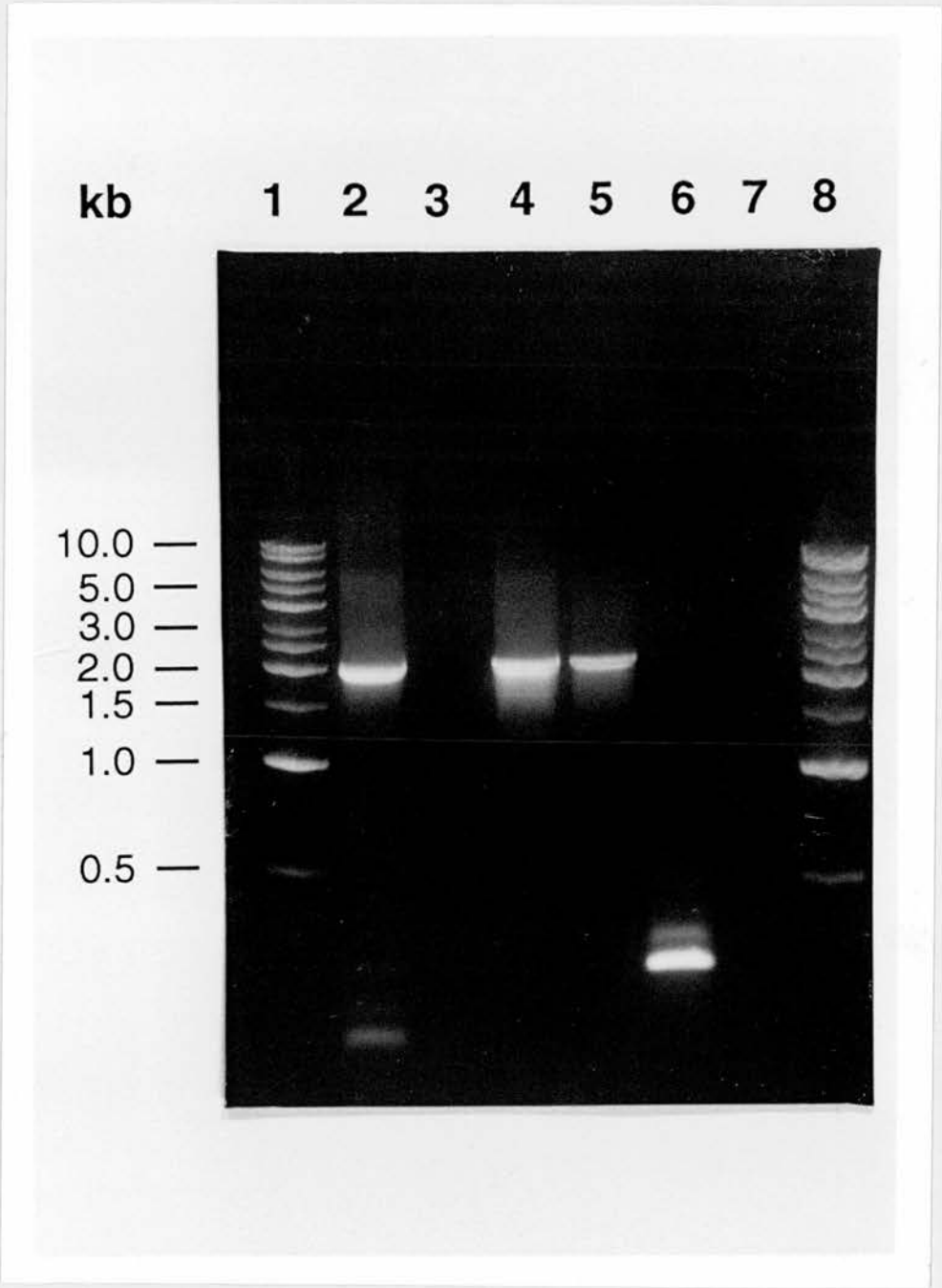

Figure 8.5 Agarose electrophoresis of the PCR product from the PCR fragment sub-cloned into the plasmid vector pCR II. Lane 2 contains the original PCR product; lanes 3-5, the PCR products from 3 positive pCR II clones; lane 6 contains the PCR product from a negative pCR II clone; and lane 7 contains the PCR product from a reaction in which no DNA template was added. The PCR product from the sub-cloned pCR II clones, in lanes 4 and 5 was slightly larger than the original PCR product (lane 2) due to the presence of extra vector sequence. Lanes 1 and 8 contain DNA size markers.

```
NH2.....Glu Gly Xxx Xxx Xxx Leu Ala Gly Glu Val Xxx Asn Lys Gly Ala Lys Gly
5' .....GAG GGG BCC CGH GRH CTG GCT GGA GAG GTT GGY AAC AAA GGA GCC AAG GGA

        Asp Arg Xxx Leu Ser Gly Pro Arg Gly Pro Gln Gly Xxx Leu Gly Glu Pro
        GAC CGA GGY TTG TCT GGA CCC AGA GGC CCC CAG GGA GHT CTT GGG GAG CCC

        Gly Lys Gln Gly Ser Arg Gly Asp Pro Xxx Asp Ala Gly Pro Arg Gly Gly
        GGA AAG CAG GGA TCT CGG GGA GAC CCC BGT GAT GCA GGA CCC CGT GGA GGC

        Ser Gly Gln Thr Gly Pro Lys Gly Asp Pro Gly Arg Pro Gly Phe Lys Tyr
        TCA GGA CAG ACA GGC CCC AAG GGA GAC CCC GGC AGG CCT GGA TTC AAA TAC

        Pro Gly.....
        CCA GGA.....

        ...........................................................
        ...........................................................

        ...........................................................
        ...........................................................

        Asn Leu Phe Pro Asn Gln Leu Pro Gly Met Asp Met Ile Lys Gln Glu Gly
        AAC CTG TTC CCA AAC CAG CTG CCT GGA ATG GAT ATG ATT AAG CAG GAG GGA

        Asp Thr Thr Arg Glu Ile Leu Leu Thr Leu Leu Lys Pro Val Ala Ser Ser
        GAC ACA ACA CGG GAA ATA TTG CTG ACA CTG CTG AAG CCA GTT GCT TCT TCA

        Ala Asp Arg Ala His Leu Leu Lys Thr Leu Pro Val Trp Arg Ala Val Ser
        GCT GAC CGG GCT CAC TTG CTC AAA ACA CTT CCA GTC TGG AGA GCT GTG TCT

        Ile Cys Phe Asn Pro Thr Asp Leu Pro Ala Gly Ser Ala Arg Ala Asp Arg
        ATT TGT TTC AAC CCA ACT GAC CTG CCA GCC GGT TCT GCT AGA GCA GAC AGG

        Pro Gly Pro Gly Ser Gln Gly Gly Val His Ser Ala Val Ala Gly Gly....COOH
        CCT GGC CCT GGT TCC CAG GGT GGC GTC CAC TCG GCT GTG GCA GGA GGA....3'
```

Figure 8.6 Partial nucleotide and deduced amino acid sequence of the antigen recognized by the anti-sp55^{sox} IgG. The figure shows the sequence of the first 212 nucleotides of the N-terminal end, and the first 258 nucleotides of the C-terminal end of the cDNA insert which was positively identified by the anti-sp55^{sox} IgG during immunoscreening of a λgt 11 testis cDNA expression library.

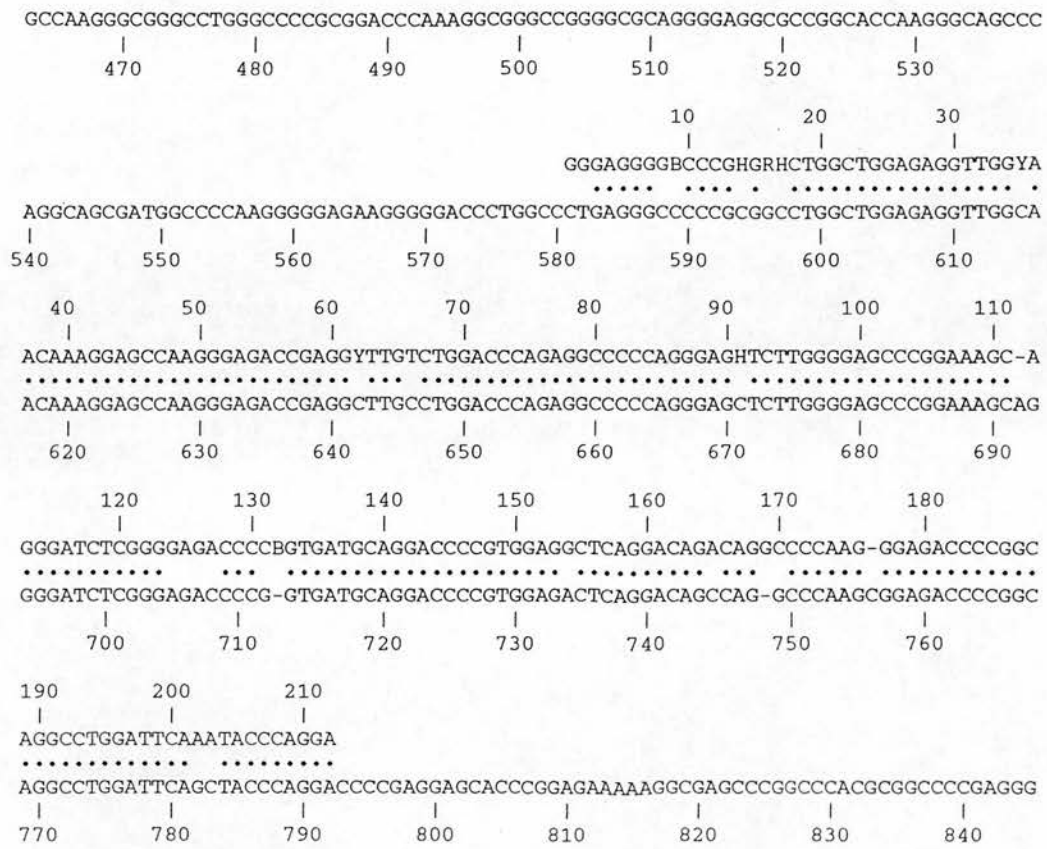

Figure 8.7 Comparison of the nucleotide sequences of the N-terminal end of the cDNA insert recognized by the anti-sp55^{sox} IgG and the alpha-2 subunit of human collagen type VI. The dots represent homologous nucleotides and the gaps indicate where no homology exists.

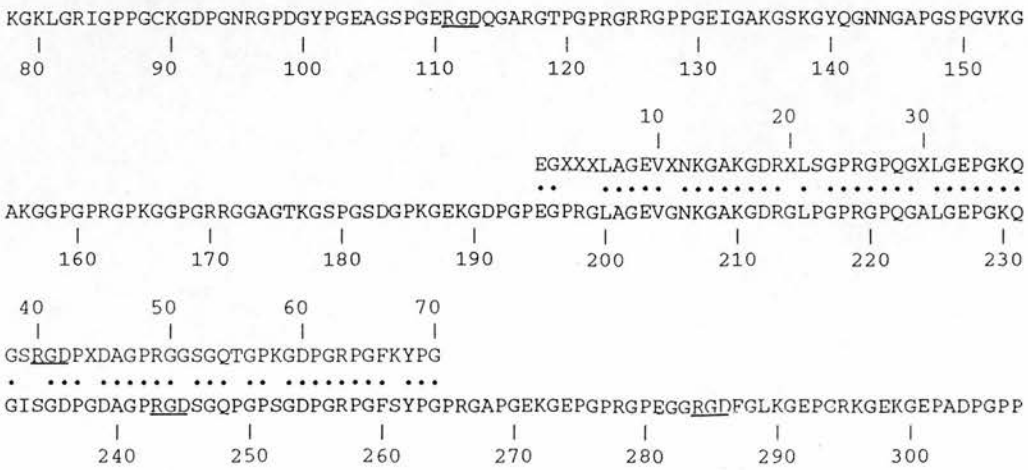

Figure 8.8 Comparison of the deduced amino acid sequence of the N-terminal end of the antigen recognized by the anti-sp55^{sox} IgG and the amino acid sequence of the alpha-2 subunit of human collagen type VI. As before, the dots represent regions of homology and the gaps indicate where no homology exists. The regions underlined denote potential cell binding domains. i.e. RGD (Arg-Gly-Asp) sequence motifs..

8.6). A sequence database (Seqnet) search was conducted using the nucleotide sequence data available, searching sense and anti-sense strands. This revealed that the Sp6/M13 R sequenced end of the DNA insert, showed a strong degree of homology with the alpha-2 subunit of human collagen type VI. The homology began at base 3 of the 5' end of the DNA insert and continued right up to base 212 of the 5' end, where the available sequence data ended (Figure 8.7) The homology between the two sequences was given a quality value of 181.2, which is indicative of very strong homology. The T7/M13 F sequenced end of the insert, the 3' end, did not show significant sequence homology with any other sequence in the Seqnet database, and the predicted amino acid sequence of this portion of the sequence is shown in Figure 8.8.

Western blot analyses of human spermatozoa with a polyclonal antibody raised against human collagen, type VI (Figure 8.9) revealed that the $sp55^{sox}$ antigen recognised by anti-$sp55^{sox}$ IgG, was also recognized, very weakly, by the anti-collagen polyclonal antibody. Likewise, the anti-$sp55^{sox}$ IgG weakly recognised the two of the subunits of human collagen, type VI. The anti-collagen antibody recognised 4 bands in human spermatozoa, 2 of which appeared to exhibit electrophoretic mobility similar to the 2 antigens (55kDa and 135kDa) targeted by the anti-$sp55^{sox}$ IgG. None of the bands were the correct size to be the sub-units of human collagen, type VI. These findings suggest that $sp55^{sox}$ contains a collagen-like domain.

8.4 Discussion

It was about 20 years ago that recombinant DNA technology was introduced as a tool for the biological sciences and has permitted the detailed analyses of individual genes and gene products in living organisms. In an attempt to characterize and identify the sperm antigen $sp55^{sox}$, molecular cloning techniques were exploited, based around the immunological screening of a human testicular cDNA library, cloned into the expression vector λgt 11.

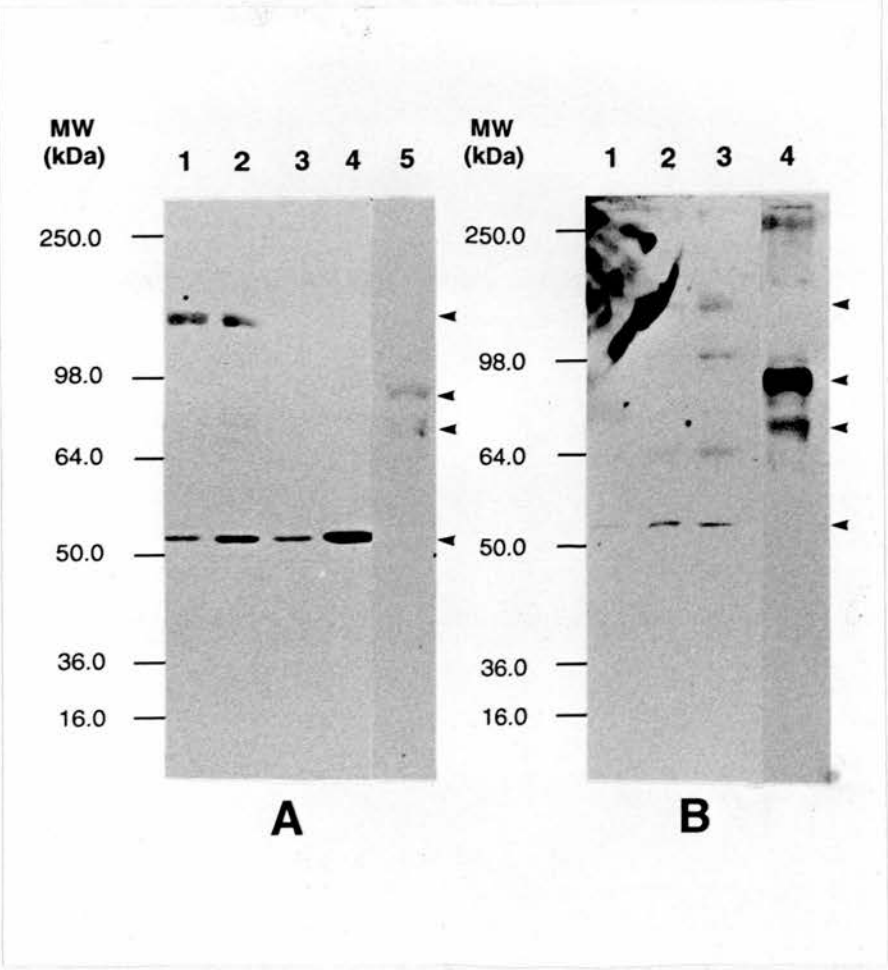

Figure 8.10 Western blots comparing the immunoreactivity of the anti-sp55^{sox} IgG and an anti-human collagen VI polyclonal antibody. SDS-solubilized human sperm protein and purified human collagen VI were subjected to SDS PAGE and then probed with the anti-sp55^{sox} IgG (panel A) and the anti-human collagen VI polyclonal antibody (panel B). Panel A: lanes 1-4 all contain SDS -solubilized human sperm (equivalent to 5 x 10^6 sperm), each from different donors; lane 5 contains human collagen type VI (10μG). Panel B: lanes 1-3 contain human sperm (5 x 10^6, from different donors); and lane 4 contains human collagen VI (10μg). The anti-sp55^{sox} IgG detected the usual bands in human sperm along with 2 of the sub-units of human collagen VI. Similarly the anti-human collagen VI polyclonal antibody detected 2 of the sub-units of collagen VI as well as several bands in human spermatozoa extracts, including a protein migrating at 55kDa . The positions of the molecular weight markers are shown to the left of each panel.

One positive clone was identified and purified, after 3 rounds of screening the library, with the anti-sp55^{sox} IgG. Amplification, sub-cloning and sequencing of the insert encoding the antigen recognised by the anti-sp55^{sox} IgG, was accomplished using the polymerase chain reaction (PCR) (Saiki *et al,* 1988). PCR was developed by a team of scientists at the Cetus Corporation in 1984. This process produces large amounts of a specific DNA fragment from a complex DNA template in a simple enzymatic reaction. The method utilizes a DNA polymerase and oligonucleotide primers (based upon known sequence of the DNA fragment of interest) to synthesize a specific DNA fragment from a single stranded template sequence. The DNA polymerase used is a heat resistant enzyme, isolated from *Thermus aquaticus,* and is thus called Taq polymerase. This enzyme allows primer annealing and extension to be carried out a elevated temperatures and thus reduces mismatching annealing to non-target sequences of DNA (Arnheim and Erlich, 1992).

Although the PCR product of the λgt11 insert could have been directly sequenced, this is not, in practice, a very efficient strategy and hence, the PCR product was sub-cloned into the plasmid vector pCR II™. Quick, and efficient sub-cloning into this vector is made possible due to the non-template dependent activity of the Taq, used in the PCR, which adds a single deoxyadenosine to the 3'-ends of all duplex molecules generated by PCR. The ends of the PCR product have 'sticky ends' (referred to earlier) which can be ligated to an appropriately digested cloning vector (Arnheim and Erlich, 1992). Sub-cloning the DNA insert into a plasmid vector has the following advantages: 1) that the plasmid is more stable during storage than phage vectors, 2) the yields from plasmid DNA purification are much higher than from phage DNA purification, and 3) the ease with which plasmid DNA can be cultured. The DNA insert was sub-cloned successfully, as shown by PCR analysis, and although the PCR product was slightly larger than the PCR

product from the phage insert, this was to be expected, due to extra vector sequence being present.

Sequence analysis of the sub-cloned DNA insert was accomplished via a protocol based upon the enzymatic method of Sanger *et al,* (1977), using a commercially available sequencing kit. This method is based again on PCR, and involves the incorporation of chain-terminating nucleotides into the amplified DNA molecules. The key to this method is the use of dideoxyribonucleoside triphosphates in which the deoxyribose 3' -OH group present in normal nucleotides, is missing. The incorporation of such a molecule into a DNA chain blocks the addition of the next nucleotide. Thus the primed *in vitro* synthesis of DNA molecules, in the presence of a minor proportion of these nucleotides, generates a mixture of DNA fragments of different lengths. These fragments can then be separated by gel electrophoresis, resulting in the formation of a DNA ladder. The fragments are differentially labelled by the incorporation of different fluorescent dyes into the 4 chain-terminating nucleotides, so that along with the terminating nucleotide, a specific dye is also incorporated into the DNA, enabling the identification of the terminating nucleotide, its' position on the DNA ladder, and hence sequencing (Alberts *et al,* 1989).

Such a strategy was employed with success in this thesis and 470 bases of insert sequence data was obtained; 212 from the 5' end of the DNA insert and 258 from the 3' end. Homology searches conducted with this data revealed that the 5' sequence showed very strong homology with the alpha-2 subunit of human collagen, type VI. It was possible that the DNA insert which was identified, sub-cloned and then sequenced was unfortunately merely a false positive picked up during the screening. However, subsequent Western blot analyses with human collagen, type VI, the anti-sp55^{sox} IgG, and a polyclonal antibody raised against human collagen, type VI, revealed that the sp55^{sox} antigen contained epitopes which were recognised by the anti-collagen

antibody, and the anti-sp55^{sox} IgG did bind to the human collagen, type VI. These findings clearly suggest that the sp55^{sox} antigen contains collagen-like domains. Indeed, there may be several proteins in human spermatozoa that also contain such domains. Unfortunately it is not possible, at this point, to determine whether or not the cDNA clone, identified during the screening process, encodes the sp55^{sox} antigen, or one of the other molecules present in human spermatozoa, that possess these collagen-like structures. However, it can be said with certainty that the selected cDNA clone does not encode for collagen type VI, itself. To begin with the sperm lysates do not contain protein with the correct molecular mass for collagen VI, and, moreover, the sequence data obtained for the 3' end of the insert did not show homology to collagen VI.

In order to determine whether or not the cDNA clone identified was in fact sp55^{sox}, further screening would have to be undertaken to identify, and isolate a full length clone of the cDNA, i.e. a clone encoding the whole protein, from the amino terminus to the carboxy terminus. The fusion protein from this clone would have to be purified, and then analysed via Western blot analysis with the anti-sp55^{sox} IgG. Once the full length clone has been isolated it should be possible to deduce the amino acid sequence of the protein and thus determine whether it is the correct size to be sp55^{sox}, although one would have to take into account any post-translational modification of the protein which may have occurred, such as glycosylation. However, the presence of possible sites for glycosylation could be determined from the amino acid sequence. Another approach would be to purify some of the sp55^{sox} antigen, in order to obtain direct amino acid sequence data for comparison with the deduced amino acid sequence of the cloned molecule. Unfortunately such work, would take a long period of time to complete, and hence could not be undertaken in the time frame of this thesis.

However, it is extremely likely that the cDNA isolated does represent a portion of sp55^{sox}, since this protein clearly does have collagen-like motifs

present, as indicated by the Western blot analyses. Collagen VI is a short chain collagen which is a heterotrimer, consisting of α1, α2, and α3 chains, with a short triple helical domain (Chu *et al,* 1988; Vuorio and De Crombrugghe, 1990). It is not uncommon for non-collagen molecules to contain collagenous domains, numerous examples existing, e.g. Clq (complement component) (Sellar *et al,* 1991), lectins (Thiel and Blake, 1989; Kawasaki *et al,* 1994), acetylcholinesterase (Krejci *et al,* 1991), and macrophage scavenger receptor (Kodama *et al,* 1990). The collagenous domains of these proteins have been shown to act as membrane anchors, and the non-collagenous domains of most of these molecules have been shown to be, very often, involved in ligand binding (Hulmes, 1995). The collagenous, triple helical domains of these molecules are characterized by the triple helices, forming X-Gly-X repeat (Hulmes, 1995), which is also present in the sequence encoding the sp55^{sox} antigen. This implies that sp55^{sox} must either be a component of the trimer which makes up the triple helices, or is itself a trimer. The deduced amino acid sequence of the collagen-like domain of sp55^{sox} also contains a possible cell binding domain. i.e. Arg-Gly-Asp (Chu *et al,* 1988), and 5 potential phosphorylation sites, i.e. 3 serine residues, 1 threonine residue and 1 tyrosine residue.

But what could the role of such a protein be, in a human sperm NADPH oxidase-like system? It is possible that the sp55^{sox} component of the system is involved in the anchoring the enzymatic components of the system to the plasma membrane of the spermatozoon, and that the non-collagenous domain of the molecule is involved in ligand-binding of some sort, as described above. Interestingly, it has been shown that cytoskeletal components are essential for activity of the NADPH oxidase of leucocytes (Woodman *et al,* 1991, Elbenna *et al,* 1994). In this instance, the molecule involved is actin, but it is possible that the collagen-like domain of sp55^{sox} fulfils a similar role. It has been shown, in bovine neutrophils, that the activity of NADPH oxidase is dependent on the

oxidases' association with actin (Sandgren *et al,* 1992). Similarly, the catalytic activity of acetylcholinesterase is dependent on the presence of a collagen-like tail sub-unit, that anchors the catalytic sub-units of the enzyme to the plasma membrane (Krejci *et al,* 1991). Thus $sp55^{sox}$, could be performing a similar role in the superoxide generating system of human sperm, i.e. associating with the oxidase at the plasma membrane and maintaining its activity. It is also possible, due to the ligand binding properties of most collagen-like proteins (Hulmes, 1995), that this protein is somehow involved directly in the regulation of oxidase activity by a particular extracellular ligand, e.g. NADPH, follicular fluid component, etc.. In order to address this possibility the entire sequence of the protein will have to be deduced and analyzed for possible ligand binding sites.

Outside a role in ROS generation, it is also possible that molecules on the surface of the spermatozoon, with collagen-like domains, are involved in preventing an immune response by the females reproductive tract, or in autoimmunity in the male. It has been shown that mannose binding protein, a molecule with a collagen-like domain, is involved in preventing auto-immune reactions, and in mediating the immune response to foreign molecules (Riddihough, 1994). It is possible that when the females reproductive tract encounters spermatozoa, or when the testis-blood barrier is broken, the presence of these ubiquitous, human-specific, collagen-like domains, on the surface of the spermatozoon may prevent the occurrence of an immune response by the female, or an auto-immune response by the male. Disorders in these molecules, leading to the collagen like domains being recognized as foreign, may be one cause of immune response-mediated infertility (Alexander, 1989; Jones, 1994).

8.5 Summary and conclusions

The partial cDNA for the gene encoding an antigen recognized by the anti-sp55^{sox} IgG was isolated from a human testicular cDNA expression library. The phage DNA was isolated, the cDNA insert amplified by PCR, and then sub-cloned in the plasmid vector, pCR II™. The insert was approximately 1.9kb in length and was partially sequenced. 212 nucleotides from the 5' -end of the insert were sequenced, along with 258 from the 3' -end. The sequence data from the 5' -end showed very strong homology to the triple-helical domain of the alpha 1 sub-unit of human collagen, type VI, although the 3' -end did not show any significant homology with collagen VI.

Western blot analyses revealed that the sp55^{sox} antigen possessed antigens recognised by a polyclonal antibody raised against human collagen, type VI, and similarly the anti-sp55^{sox} IgG recognized epitopes present in the alpha 1 and 2 sub-units of human collagen, type VI, indicating that the molecules shared similar structural domains, confirming the homology shown by sequence analyses.

In conclusion, human spermatozoa contain numerous molecules containing collagen-like domains, such molecules have not been described in human spermatozoa before. One of the molecules with a collagen like domain, sp55^{sox}, is also associated with NADPH-induced ROS generation by these cells, and is possibly involved in anchoring the enzymatic component(s), directly responsible for ROS generation, to the plasma membrane of these cells.

Chapter 9

Cross species generation of ROS by spermatozoa and its' ontogeny during epididymal maturation and spermiogenesis.

9.1 Introduction

ROS generation by human spermatozoa is clearly a very important aspect of their biochemistry. It has profound implications for the control of human sperm function, both in the physiological sense and in the context of the dysfunction, associated with male infertility (Aitken and Clarkson, 1987a and b; Aitken, 1994a and b, Aitken *et al*, 1994b; de Lamirande and Gagnon, 1995; de Lamirande and Gagnon, 1993a and b). With this in mind, it would be interesting, and useful, to study ROS generation by the spermatozoa of animal species other than man. Determination of the prevalence of the ability to generate ROS across the species, would give some indication as to the biological importance and significance of this phenomenon. If generation of ROS by spermatozoa is a widely conserved feature of these cells, then it could indicate a fundamental role for this activity in normal sperm function. Investigations into ROS generation by spermatozoa, other than human, would also provide helpful information regarding the control mechanisms of this activity. Unlike human spermatozoa, the spermatozoa of other mammalian species are very uniform in their morphology and behaviour, and so it may be possible to determine whether or not the ability of human spermatozoa to generate ROS is, in any way, related to the fact that the human ejaculate generally contains significant numbers of 'abnormal' spermatozoa (World Health Organisation, 1992), especially those characterized by an abnormal, and excessive retention of cytoplasm, and thus elevated levels of cytoplasmic enzymes (Huszar and Vigue, 1988; Gavella and Lipovac, 1992; Casano *et al*,

1991; Orlando *et al,* 1994; Aitken *et al,* 1994b), including those of the HMS responsible for NADPH generation, and shown to be strongly correlated with the levels of possible ROS generation (Aitken *et al,* 1994b). Moreover, the confounding effects of leucocyte contamination on the assessment of ROS generation by suspensions of human spermatozoa, could be avoided by the use of an animal model.

Identification of an appropriate animal model would also enable analysis of the ontogeny of ROS generation by spermatozoa during epididymal maturation, and during spermiogenesis. This is obviously not a feasible, or practical option when dealing with human spermatozoa. Through such studies it may be possible to determine the point in germ cell differentiation when ROS generation first appears, and develop models for the oxidative stress observed in male sub-fertility. With such models, a systematic examination of different anti-oxidant strategies could be undertaken with the aim of preventing peroxidative damage associated sperm dysfunction. The development of an animal model to study ROS generation by mammalian spermatozoa would also facilitate investigations into the biological role of ROS in sperm function and fertilization. Finally, it would permit the assessment of this phenomenon as a possible target for contraceptive development, possibly through the induction of active immunity against key components of the ROS generating system, or via a pharmacological approach. Such strategies could be investigated provided that the ROS generation system present in human spermatozoa, expresses features that are unique, and not shared by any other ROS generating systems, such as the leucocyte NADPH oxidase.

From the literature, it is apparent that the spermatozoa of species other than the human do indeed generate ROS. As long ago as 1946, Tosic and Walton showed that bovine spermatozoa generated hydrogen peroxide (Tosic and Walton, 1946) and more contemporary analyses have shown that rabbit spermatozoa (Holland *et al,* 1982), murine spermatozoa (Alvarez and Storey,

1984), rat spermatozoa (Kumar *et al,* 1990), and hamster spermatozoa (Bize and Sharpe, 1990) all generate ROS. However, in all cases the cases mentioned above, an NADPH oxidase-like enzyme complex has not been implicated, and it is only in bull spermatozoa and rabbit spermatozoa that the probable mechanisms of ROS generation have been determined, or even investigated. Bull spermatozoa appear to generate ROS via the oxidative deamination of aromatic amino acids (Tosic and Walton, 1950), whilst in rabbit spermatozoa, generation of ROS is derived from leakage of electrons from the mitochondrial electron transport chain (Killian *et al,* 1985). Neither of these mechanisms appear to be involved in the generation of ROS by human spermatozoa (Aitken and Clarkson, 1987a). Since the cellular mechanisms responsible for ROS generation by the spermatozoa of such species as the mouse, rat, hamster and guinea pig have not been previously investigated, an analysis of this activity was undertaken.

As has already been mentioned, one of the benefits of using an animal model in studies of ROS generation is the potential to study the ontogeny of the ROS generating system during epididymal maturation. The epididymis is a single, highly convoluted duct, that is closely associated with the surface of the testis, extending from the anterior, to the posterior pole of that organ (Setchell and Brooks, 1988). The epididymis is held in contact with the testis via connective tissue; it is coiled into relatively discreet segments and contained within a fibrous tissue capsule. The segments of the epididymis can loosely be divided into four parts; the initial segment, into which the efferent duct of the testis empties, the caput (head), corpus (body), and cauda (tail), as shown diagramatically in Figure 9.1. There is an alternative system of nomenclature based upon histological and functional criteria (Glover and Nicander, 1971), but for the purpose of this study the more traditional, anatomical criteria for the sub-divisioning of this organ will be used.

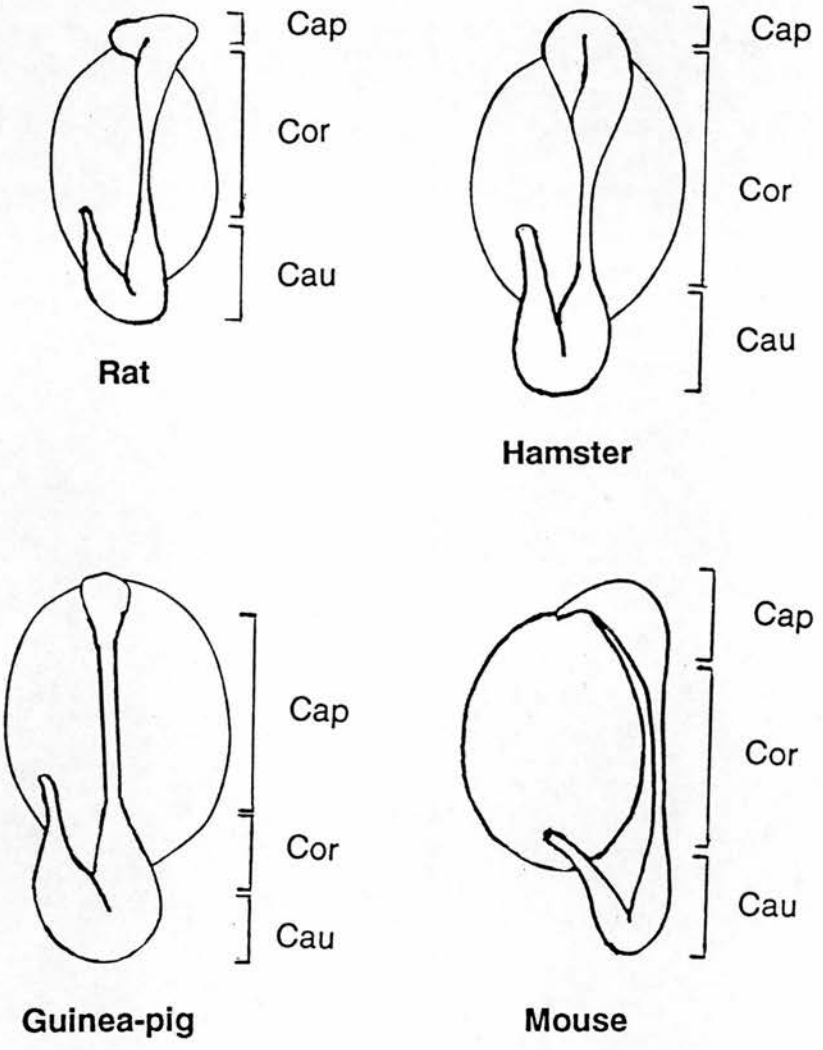

Figure 9.1 Diagrammatic representation of the anatomical regions of the epididymides of various mammalian species. Cap - caput epididymis; Cor - corpus epididymis; and Cau - cauda epididymis

It has been shown that in most mammalian species the epididymis plays an indispensable role in the acquisition of sperm fertilizing capacity. Spermatozoa leaving the testis, although being highly differentiated, remain incapable of fertilizing an ovum without assistance (Bedford, 1975). Although spermatozoa, at this point, have lost most if not all ability for protein and lipid biosynthesis, they are equipped to modify and degrade existing molecules (Bedford and Hoskins, 1990), and also the biochemical environment of the lumen of the epididymis and the cells of the epididymal epithelium can profoundly alter the biochemical, and thus, the biological status of the maturing spermatozoon (Moore *et al,* 1989; Cooper, 1990). During their passage through the epididymis, spermatozoa undergo many morphological and biochemical changes which enable them to successfully traverse the female reproductive tract, recognise, bind and then fuse with the egg, and thus effect fertilization (Amann *et al,* 1993). The maturational changes that the spermatozoon undergoes during its' transit through the epididymis are not very well understood or characterised, although it has long been appreciated that the changes are not merely the result of a passive ageing process, but that, in fact, the maturational events associated with the epididymis are an actively orchestrated series of cellular events, which are very carefully regulated (Robaire and Hermo, 1988). However, epididymal maturation of spermatozoa, is a very complicated phenomenon, with many practical difficulties existing which hinder its' study, and thus, it remains a very poorly understood process. These maturational events include:

1) Alterations in metabolism (Mann and Letwak-Mann, 1981)

2) Changes in movement characteristics (Acott *et al,* 1983)

3) Changes in zona binding ability (Saling, 1982; Moore *et al,* 1983; Moore, 1990)

4) Changes in intracellular ions (Vijayyaraghavan and Hoskins, 1989).

5) Plasma membrane associated changes (see below)

Plasma membrane associated changes include alterations in the distribution and composition of phospholipids in the sperm plasma membrane (Rana *et al,* 1993); changes in the surface charge of the spermatozoon, such that the cells become increasingly negatively charged as they pass through the epididymis (Bedford, 1963; Bedford *et al,* 1973; Moore, 1979); and changes in the pattern of glycosylation expressed by the glycolipid and glycoproteins of the sperm plasma membrane (Tulsiani *et al,* 1993). Another fundamental change during epididymal transit is the progressive formation of structurally stabilizing disulphide bonds in the nucleus and tail of the spermatozoon (Calvin and Bedford, 1971).

In most mammalian species, these maturational changes occur in the caput and corpus regions of the epididymis, with the cauda epididymis acting as a sperm reservoir for the storage and maintenance of spermatozoa, prior to ejaculation. In the human, the situation appears to be more complex, and the role(s) of the epididymis in human sperm maturation and storage remain to be fully elucidated. However, it is known that in the human the cauda epididymis cannot really be classed as a storage site for fully matured spermatozoa, as it does appear to contain relatively few sperm cells, in compared with other mammalian species (Amann *et al,* 1993).

From the foregoing, it can be appreciated that epididymal maturation has a profound impact on the functional competence of the spermatozoon, and it is not unreasonable to assume that the process may have a significant effect on the activity of key enzymes such as those responsible for ROS generation. Much data is available pertaining to the variation of enzyme activity during epididymal maturation (Hoskins *et al,* 1975; Chang and Zirkin, 1978; Kumar *et*

al, 1990; Peltola *et al,* 1992; Rigaudière *et al,* 1992; Perry *et al,* 1993; Tulsiani *et al,* 1993). Furthermore, there is even some evidence to indicate that the levels of ROS generation vary during epididymal transit. Thus, Kumar *et al* (1990), studying superoxide anion generation by rat spermatozoa, presented data indicating that superoxide anion generation by these cells increased as they passed through the epididymis, i.e. during epididymal maturation. These authors also presented data in the same paper suggesting that the levels of the superoxide scavenging enzyme, superoxide dismutase, decreased in the spermatozoa as epididymal maturation progressed. There are quite a few other reports showing changes in the levels of various antioxidant enzymes during spermatogenesis, epididymal maturation and in the different areas of the testis and epididymis (Nonogaki *et al,* 1992; Peltola *et al,* 1992; Rigaudière *et al,* 1992; Perry *et al,* 1993)

It has been suggested that the high levels of ROS generation, observed in some human sperm samples may be a consequence of incomplete sperm maturation (Aitken *et al,* 1994b). These authors have shown that an increased capacity to generate ROS is significantly correlated with the levels of cytoplasmic enzymes in the spermatozoa of such samples. The latter is thought to reflect defects during spermiogenesis, as a consequence of which, the spermatozoa retain excess residual cytoplasm (Russell, 1979). It has, in fact, been shown that defective human spermatozoa with abnormal morphologies indicative of excess residual cytoplasm, do contain greater levels of cytoplasmic enzymes, and that these cells are dysfunctional (Huszar and Vigue, 1993). Together, these results would suggest that the ability to generate high levels of ROS, in humans at least, is related to the level of maturity of the cells present in the sperm suspension and the amount of cytoplasm they contain. This raises the possibility that the pathological conditions associated with excessive ROS are a result of the presence of spermatozoa which are either not functionally mature or fully differentiated, and/or infiltration of the

ejaculate with germ cells (Aitken *et al,* 1994a). One way to address this issue would be to investigate spermatozoa that were not fully mature, e.g. epididymal and testicular spermatozoa, and their abilities to generate ROS. This is not possible in the human, but is possible in animal models. Also using animal models, experiments could be conducted to assess the role and contribution of germ cells in excessive ROS generation.

Post-testicular spermatozoa are thought to have little or no biosynthetic capability and therefore, cannot synthesise proteins and lipids, and it is during spermiogenesis that the spermatid loses it ability for *de novo* synthesis (Bedford and Hoskins, 1990). It is also during spermiogenesis that germ cells discard most of their cytoplasm (Russell, 1979). With these features of spermiogenesis in mind, it can be appreciated that such changes may have profound effects on the ability of the germ cell to generate ROS, either leading to an increase or decrease in their ability to do so. The acquisition of this information would be very informative in addressing the hypothesis that it is maturationally compromised spermatozoa, or infiltrating germ cells which are responsible for excessive ROS generation in some ejaculates.

Another, burgeoning field in male reproduction research is that of the impact of oxidative stress in the testis, and its effect on spermatogenesis, and male fertility as a whole (Cummins *et al.* 1994). It has been known for many years, and very significantly in the context of this thesis, that normal testicular function depends upon the presence of vitamin E; a major antioxidant in the defence against lipid peroxidation (Wu *et al,* 1973). The testis may be in a very delicate state of oxidative balance since there are numerous reports of it containing a battery of powerful antioxidants, including superoxide dismutase, catalase, glutathione peroxidase and glutathione transferase, (Nonogaki *et al,* 1992; Peltola *et al,* 1992; Peltola *et al,* 1994; Perry *et al,* 1993; Veri *et al,* 1993; Veri *et al,* 1994). Moreover, testes deficient in such molecules have been shown to undergo increased lipid peroxidation, and become dysfunctional.

Toxicological studies have shown that administration of certain toxicants, e.g. to laboratory animals, results in depletion of antioxidant reserves in the tissues of the testis, and increases in the peroxidative damage sustained by this organ (Al-Bayati *et al,* 1988; Peltola *et al,* 1994). Physiological roles for ROS in the testis have also been suggested, e.g. the paracrine regulation of Leydig cell steroidogenesis (Stocco *et al,* 1993) and the regulation of cell differentiation (Peltola, *et al,* 1992; Peltola et al. 1994; Jow *et al,* 1993). Thus, the balance of oxidant : antioxidant activity in the testis may be an extremely important factor in the maintenance of normal testicular function. In this context, elucidating the possible contribution of pre-cursor germ cells to the oxidative stress experienced by this organ would be very valuable.

The work described in this chapter focuses on the influence of epididymal maturation, on the ability of spermatozoa to generate ROS, both spontaneously, and in response to exogenous NADPH, PMA, and the divalent cation ionophore A23187. The animals used in these experiments were the rat, guinea pig, golden hamster, and mouse. In the case of the rat, this study was extended to cover precursor germ cells (Pachytene spermatocytes and spermatids) of the testis.

9.2 Materials and methods

All experiments were performed at least three times and the results expressed as independent, representative, longitudinal analyses, i.e. individual traces of ROS generation, or as the means of repeated measurements, with analysis of variance (ANOVA) statistical analyses being performed, to determine the statistical significance, if any, of the results obtained.

9.2.1 Animals and management

The animals used in the experiments described below, were all housed and maintained under conventional, controlled conditions in the Centre for Reproductive Biology animal house. All of the animals used were adult males and were killed by CO_2 gas inhalation, followed by cervical dislocation. The animals used were the Wistar rat, the guinea pig, the golden hamster, and the mouse.

9.2.2 Epididymal dissection and spermatozoa preparation

Immediately after killing by CO_2 inhalation and cervical dislocation, the testes and attached epididymides were excised from the animals, the connective tissue attaching the epididymides to the testis removed, and the epididymides detached. Any blood vessels present were carefully dissected from the epididymis, and the intact structure was then rinsed in BWW, and dissected into cauda, corpus, and caput regions, as indicated in Figure 9.1. The 3 different regions of the epididymis were then rinsed and placed into individual petri dishes, containing a 500µl droplet of BWW under liquid paraffin.

The cauda epididymis consisted of easily distinguishable, highly convoluted tubules, containing very high numbers of spermatozoa. To collect the spermatozoa the tubules were punctured, under liquid paraffin, and then gently squeezed with forceps. This resulted in the spermatozoa being extruded from the cauda epididymis, as dense 'strings' of compacted spermatozoa that were gently guided into a 500ml droplet of BWW, with a heat-sealed, glass hook. The spermatozoa were then allowed to disperse in the droplet for approximately 30 minutes, before they were sampled and counted.

The spermatozoa from the corpus and caput portions of the epididymis were not so easily retrieved. The corpus epididymis is merely a very fine, single tubule, which does not contain great numbers of spermatozoa. In order to collect the maximum number of spermatozoa from this portion of the

epididymis, the tissue was repeatedly cut with very fine scissors, and the spermatozoa allowed to diffuse out, into the BWW droplet. The same strategy was employed in the case of the caput epididymis. Once the spermatozoa appeared to have completely diffused out of the epididymal segments, the tissue was removed from the BWW droplet and discarded. The sperm suspensions were then sampled and counted.

The various sperm populations were then washed in fresh BWW by centrifugation at 1,000rpm for 10 mins and resuspended in BWW to give a resulting cell concentrations of 5 x 10^6 cells/ ml. The motilities of all samples were then assessed and the percentage of cells with cytoplasmic droplets recorded.

9.2.3 Preparation of rat testicular germ cells.

The preparation of rat testicular germ cells was kindly carried out by Dr Tony West, and his assistance in preparing material for this study is gratefully acknowledged. The protocol followed was similar to the one described by Meistrich *et al* (1981), as modified by West *et al* (1994). Briefly, adult Wistar rats were killed as described above, and the testes recovered within 1-2 minutes of death. The testes were chopped into $2mm^3$ pieces and dispersed via two, 30 minute incubations in an enzyme solution (enzyme solution 1) containing 0.1% w/v collagenase (Worthington Biochemical, Freehold, NJ, USA), 0.2% w/v hyaluronidase (Sigma), 0.03% v/v trypsin inhibitor (Sigma), all in a medium (medium A) containing M199 medium (Gibco), 25mM HEPES, pH 7.4 (Gibco), 0.1% w/v BSA, 0.03% w/v DNase, 0.2% w/v glucose (Sigma), penicillin and streptomycin, both at 10,000U/ml (Gibco). Any tissue remaining undispersed at this point was removed and was subjected to a further 30 minute incubation in enzyme solution 2, which was enzyme solution 1 with 200U Dispase (Collaborative Biomedical, Bedford, MA, USA). The cell suspensions from the two enzymatic incubations were pooled and filtered through a 60µm gauze

mesh. The resultant cell suspension was pelleted at 800 *g* for 10 minutes and resuspended in 10ml of medium B, which was the same as medium A with the following modifications; 0.5% w/v BSA instead of 0.1% and the added constituents EDTA (1mM) and heparin (12,500U) (Sigma). This cell suspension was then loaded onto an elutriation rotor in medium B and fractions collected according to cell size as follows; < 8μm (F1), 8-9μm (F2), 10-13μm , 14-15μm (F3), 16-20μM and > 20μM (F4). Samples of each fraction were examined microscopically and their respective compositions are shown in Table 9.1.

Table 9.1 Cellular composition of elutriation fractions of rat testicular cells

Elutriation Fraction	Cell sizes	Most abundant cell type
1	< 8μm	elongate spermatids
2	8-9μm	round spermatids
3	10-13μm, 14-15μm	Pachytene spermatocytes
4	16-20μm, > 20μm	Sertoli cells

In the studies carried out here, only fractions 1-3 were used. The concentration of cells present in each of the fractions 1-3 was determined using an improved Neubauer haemocytometer as described in the general Material and Methods section of this thesis. The cell suspensions were then pelleted at 800 *g* for 10 minutes and resuspended in BWW to give a resulting cell concentration of 5×10^6 cells/ ml.

9.2.4 ROS generation

ROS generation by the epididymal spermatozoa and germ cells was monitored via lucigenin and luminol-dependent chemiluminescence as described earlier. All cell suspensions were subjected to the FMLP 'challenge test' before use, and any suspensions which responded positively to this test were discarded and not used for any further analyses.

In all experiments, 400µl of the cell suspensions, at a concentration of 5 x 10^6 cells/ml, were used. Spontaneous ROS generation by the cells was monitored by lucigenin and HRP-enhanced, luminol-dependent chemiluminescence, whilst exogenous NADPH-induced superoxide anion generation was monitored solely employing lucigenin. The cells were also stimulated to generate ROS in response to PMA and the divalent cation ionophore, A23187, and the ROS generated in response to these two reagents were monitored by HRP-enhanced, luminol-dependent chemiluminescence.

In each experiment, the chemiluminescence was monitored for around 20 minutes and then the appropriate stimulus added. As described previously, NADPH was used at a final concentration of 500µM, and PMA at a final concentration of 100nM. A23187 was purchased from Calbiochem and made up as a 10mM stock solution in DMSO, which was stored at -20°C. Secondary stock solutions of the A23187 were made up at a concentration of 1mM by diluting the DMSO stock preparation 1:10 with medium BWW. This preparation was stored for at least 3 days, at 4°C, before use. On the day of the experiment this 1mM stock was further diluted to 250µM with medium BWW. 4µl of this solution was added to the sperm suspension, at the appropriate time, to give a final working concentration of 2.5µM A23187.

After addition of the various agonists to the sperm suspensions, ROS generation was monitored for a further 40 minutes. In some instances, to confirm the identity of the ROS being monitored by HRP-enhanced, luminol-dependent chemiluminescence, 500U of catalase (bovine liver, Calbiochem) was added.

The results from these experiments were expressed as representative, individual luminometer traces, and as the means ±S.E. of 3 separate experiments. The mean values shown, were obtained using the integrated number of counts over the five minute period immediately after addition of the stimulatory reagent, as described in earlier chapters of this thesis. Analysis of variance (ANOVA) statistical tests were carried out in order to determine the statistical significance of the results obtained, and the Fisher least significant difference (FLSD) computed at the 0.05 level of significance.

9.3 Results

9.3.1 Characterization of epididymal spermatozoa

In general, the epididymal spermatozoa of the different species employed, showed marked similarities in their crude, physical characteristics, i.e. motility and the prevalence of cytoplasmic droplets. The mean data from these observations are shown in table 9.2. The percentage of motile spermatozoa in the cell suspensions increased as the cells progressed through the caput to the cauda epididymis., and this trend was statistically significant in the guinea pig and hamster ($P < 0.05$).

The percentage of cells with a retained cytoplasmic droplet, in suspensions from each of the three epididymal regions, showed a reversed trend, i.e. caput > corpus > cauda, in all species except the guinea pig whose epididymal spermatozoa did not appear to have retained cytoplasmic droplets in any region of the epididymis. In the three other species the cauda spermatozoa had a significantly lower percentage of cells with cytoplasmic droplets than spermatozoa from the corpus or caput epididymis ($P < 0.05$).

Table 9.2 Characteristics of epididymal spermatozoa

Species	Epididymal region	% Motility (mean ± S.E.)	% Cytoplasmic droplets (mean ± S.E.)
Guinea pig	Cauda	68.0 (± 2.646)	0
	Corpus	55.2 (± 1.114)	0
	Caput	7.0 (± 6.506)	0
Golden hamster	Cauda	69.0 (± 10.017)	23.53 (± 6.635)
	Corpus	46.7 (± 5.696)	57.67 (± 6.936)
	Caput	15.0 (± 3.464)	67.73 (± 3.267)
Mouse	Cauda	67.33 (± 4.631)	14.67 (± 8.373)
	Corpus	49.67 (± 7.688)	39.67 (± 8.413)
	Caput	26.01 (± 6.936)	45.00 (± 15.275)
Rat	Cauda	60.07 (± 7.874)	18.90 (± 11.2)
	Corpus	55.10 (± 6.523)	54.73 (± 10.641)
	Caput	34.47 (± 1.737)	72.23 (± 4.485)

9.3.2 ROS generation by epididymal spermatozoa.

Epididymal spermatozoa, and testicular germ cells, did exhibit the capacity to generate ROS, as monitored by lucigenin and luminol-dependent chemiluminescence, although the levels of the activity varied with the conditions under which it was monitored.

Spontaneous ROS generation

The spermatozoa from all regions of the epididymis, and of all the species tested, did appear to spontaneously generate the superoxide anion, as monitored by lucigenin-dependent chemiluminescence (Figures 9.2a and 9.6a, 9.10a, 9.14a, and 9.22). This activity did not vary significantly vary between the different epididymal regions or between species.

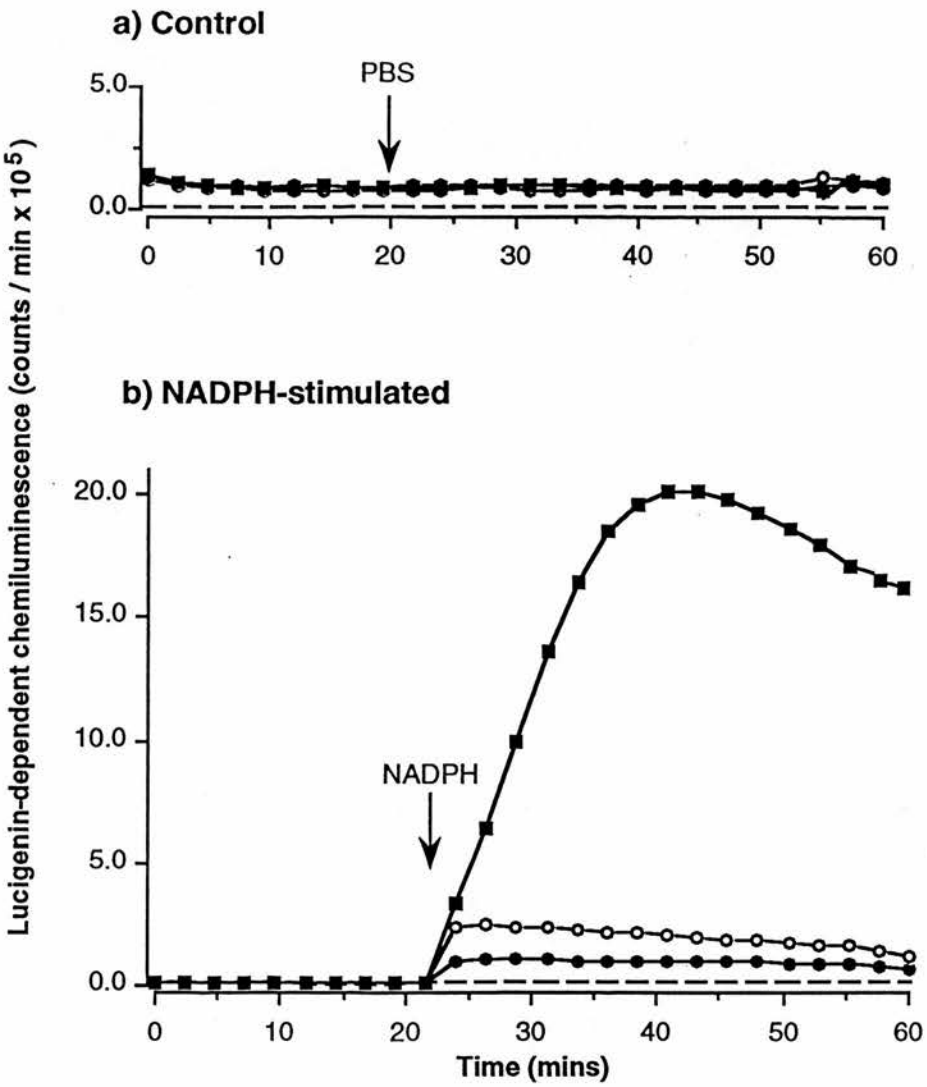

Figure 9.2 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by guinea pig epididymal spermatozoa.
a) Control chemiluminescence; b) NADPH-stimulated chemiluminescence. Experiments were carried out in triplicate, and the traces are representative examples. Caput spermatozoa =——■——; corpus spermatozoa=——o—— ; cauda spermatozoa= ——●—— ;
BWW control = - - - - - .

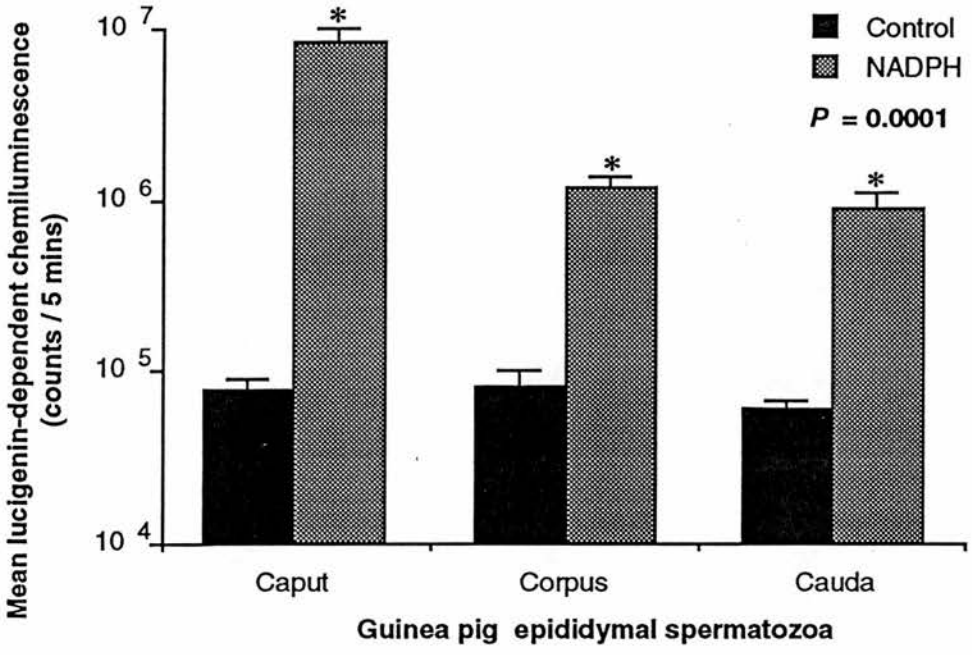

Figure 9.3 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by guinea pig epididymal spermatozoa. Results are the means ± S.E. of 3 separate experiments. The NADPH induced reponses were significantly higher than control reponses, $*P < 0.05$. Overall, the reponses were significantly different from one another ($P = 0.0001$). Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

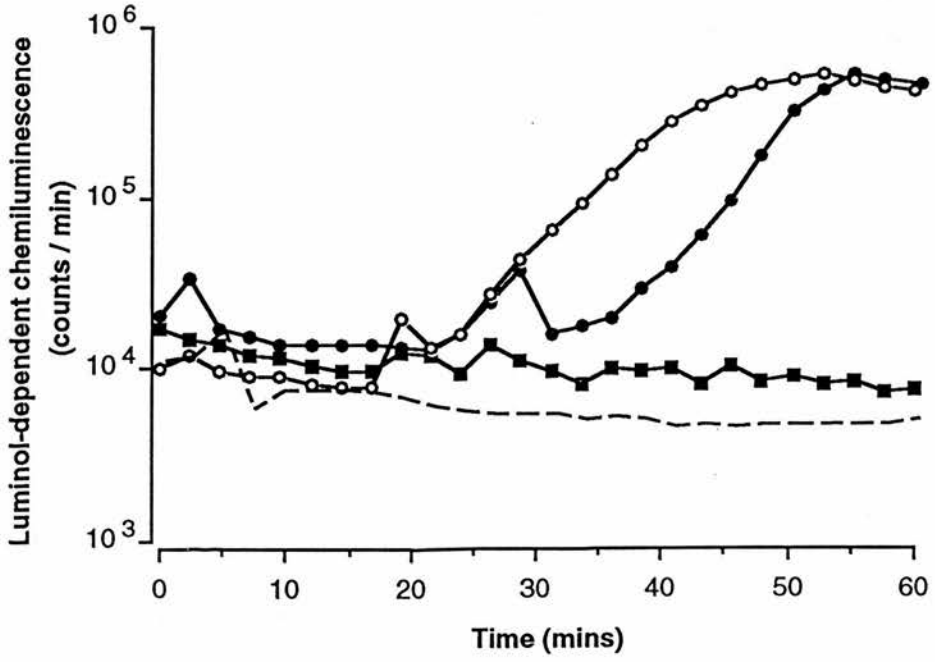

Figure 9.4 Spontaneous HRP-enhanced, luminol-dependent chemiluminescence by guinea pig epididymal spermatozoa. Experiments were carried out in triplicate, and the traces are representative examples.
Caput = —■—; Corpus = —o— ; Cauda = —●— ; and BWW control = - - - - - .

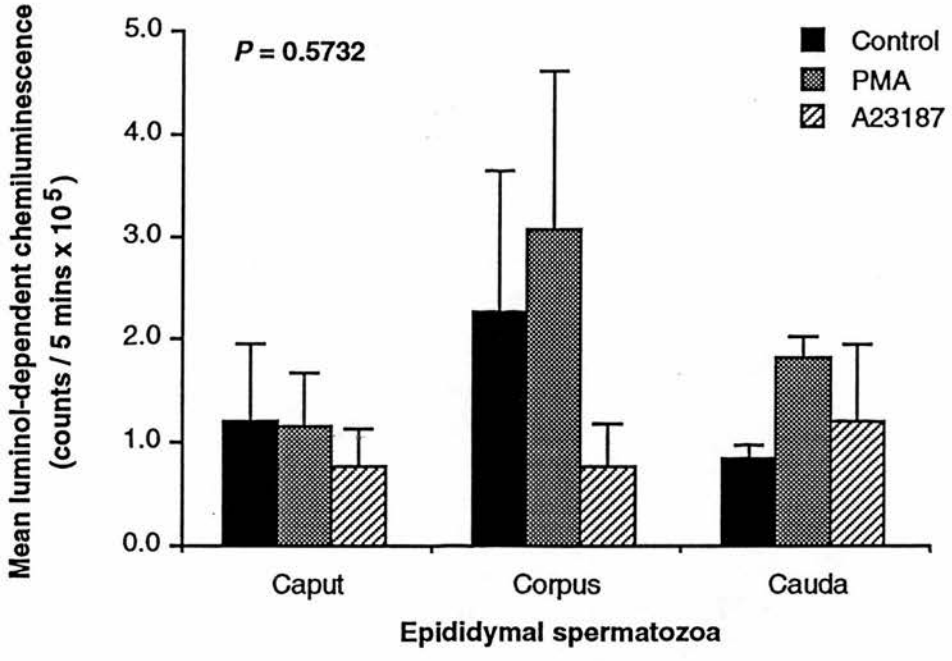

Figure 9.5 Effect of PMA (100nM) and A23187 (2.5µM) on luminol-dependent chemiluminescence by guinea pig epididymal spermatozoa. Results are the means ± S.E. of 3 separate experiments. No spermatozoa showed a significant response to PMA or A23187, and overall, the results were not significantly different from one another.

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

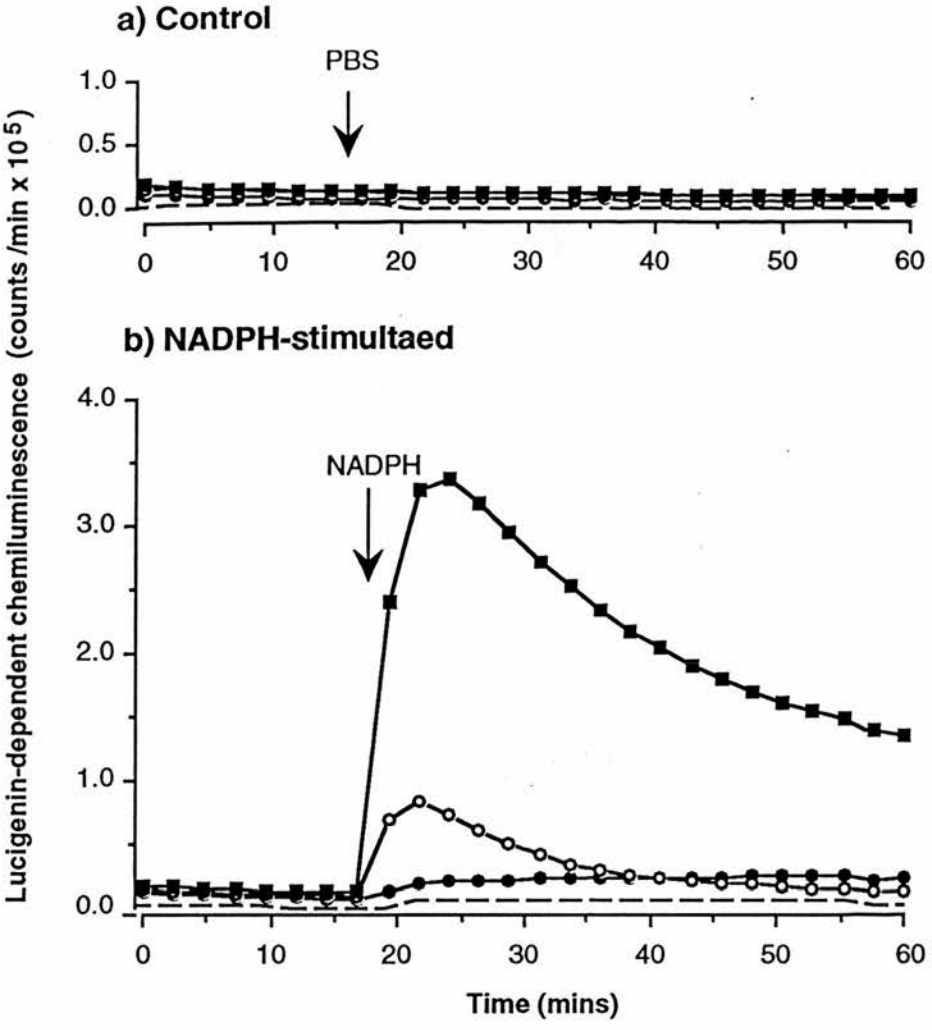

Figure 9.6 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by hamster epididymal spermatozoa. a) Control chemiluminescence; b) NADPH-stimulated chemiluminescence. Experiments were carried out in triplicate, and the traces are representative examples. Caput spermatozoa =—■—; corpus spermatozoa =—○— ; cauda spermatozoa = —●— ; and BWW control = - - - - - .

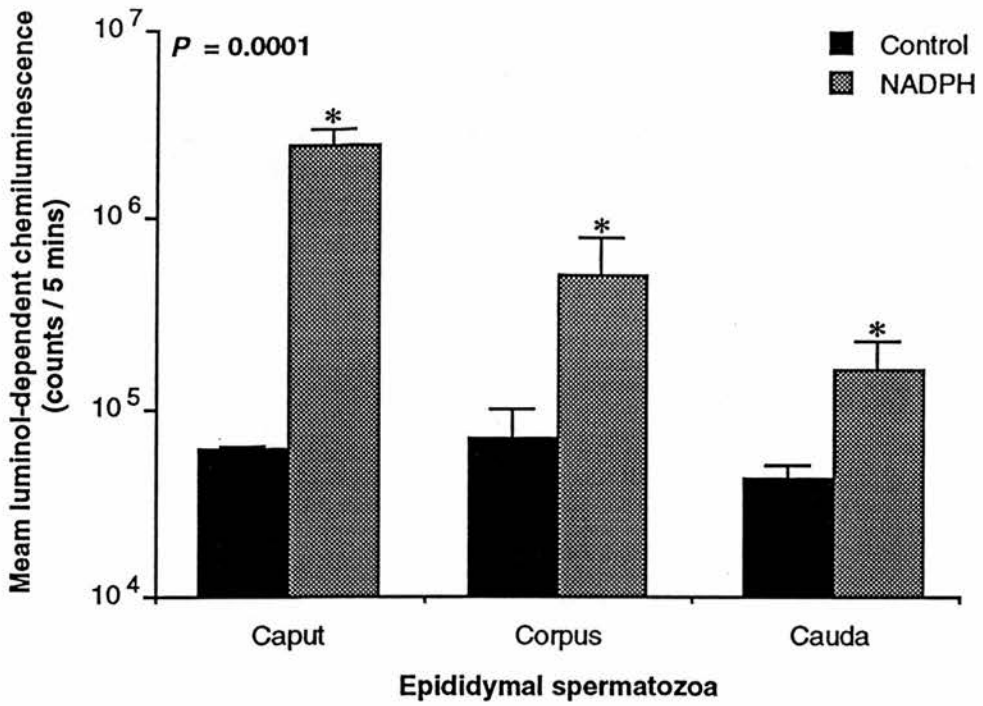

Figure 9.7 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by hamster epididymal spermatozoa. Results are the means ± S.E. of 3 separate experiments. The NADPH-induced responses were significantly higher than control responses, * $P<0.05$. Overall, the reponses were significantly different from one another (P = 0.0001). Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

Epididymal spermatozoa did spontaneously generate hydrogen peroxide, as monitored by HRP-enhanced, luminol-dependent chemiluminescence (Figures 9.4, 9.8, 9.12, 9.16 and 9.23). The levels of ROS produced did vary between the species and also between epididymal regions. In all species a trend was observed regarding spontaneous hydrogen peroxide production, with the levels of hydrogen peroxide generation increasing as spermatozoa progressed from the caput epididymis through to the cauda (Figure 9.23), and this trend was significant in the hamster and rat ($P < 0.05$).

Exogenous NADPH-induced ROS generation

Epididymal spermatozoa did generate the superoxide anion in response to exogenous NADPH, as monitored by lucigenin-dependent chemiluminescence, although the magnitude of the response did vary between species, and also between the various epididymal regions (Figures 9.2b, 9.3, 9.6b, 9.7, 9.10b, 9.11, 9.14b, 9.15, and 9.24). Exogenous, NADPH-induced superoxide generation by the epididymal spermatozoa of all species varied in its' magnitude and was dependent on the epididymal maturational status of the spermatozoa. The general trend observed was that of a decrease in the response to NADPH, as the spermatozoa progressed from the caput epididymis through to the cauda epididymis. In some species, the variation in the levels of NADPH-induced superoxide by the spermatozoa from the different epididymal regions was very pronounced. For example, in the rat only caput spermatozoa were induced to generate increased levels of the superoxide anion upon addition of NADPH (Figures 9.14b, 9.15, and 9.24).

PMA and A23187-induced ROS generation

Neither PMA or A23187 significantly stimulated levels of ROS generation higher than control levels, by any of the spermatozoa of the various species investigated (Figures 9.5, 9.9, 9.13, and 9.17).

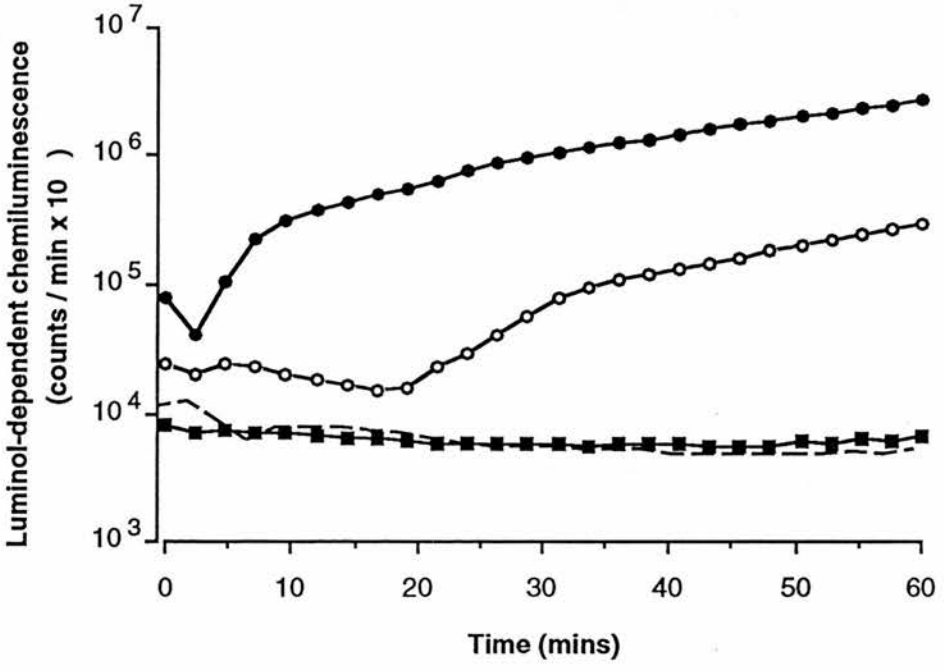

Figure 9.8 Spontaneous HRP-enhanced, luminol-dependent chemiluminescence by hamster epididymal spermatozoa. Experiments were carried out in triplicate, and the traces are representative examples.
Caput = —■—; Corpus = —o— ; Cauda = —●— ; and BWW control = - - - - - .

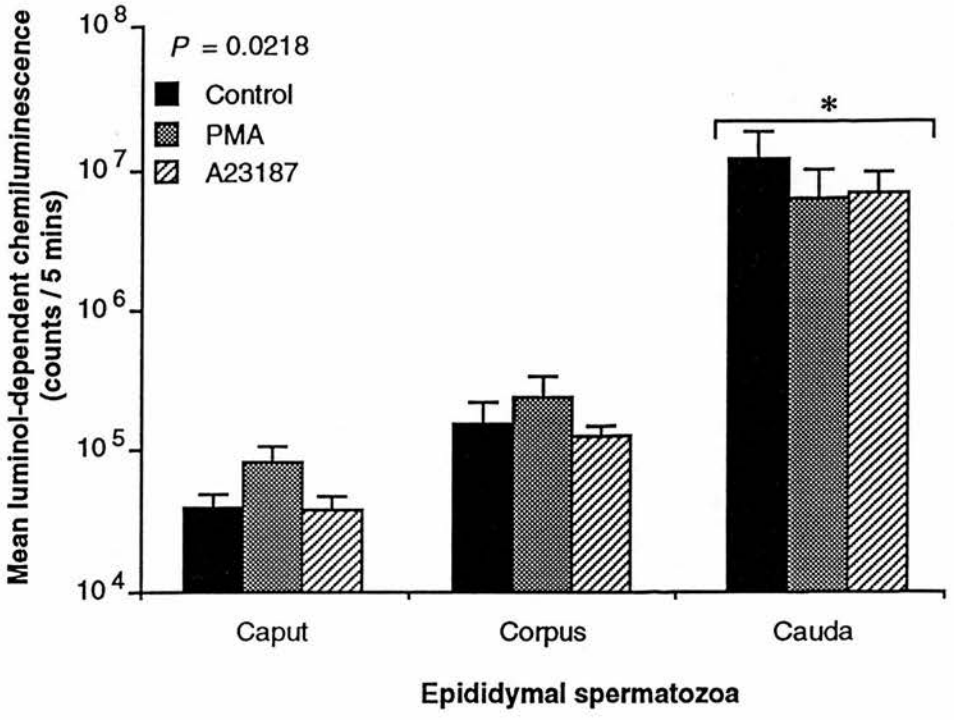

Figure 9.9 Effect of PMA (100nM) and A23187 (2.5μM) on luminol-dependent chemiluminescence by hamster epididymal spermatozoa. Results are the means ± S.E. of 3 separate experiments. There were no significant reponses to PMA or A23187, but luminol-dependent chemiluminescence by cauda spermatozoa was significantly higher than that by spermatozoa from the other regions of the epididymis, $*P < 0.05$. Overall, the results were significantly different from one another ($P = 0.0218$).

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

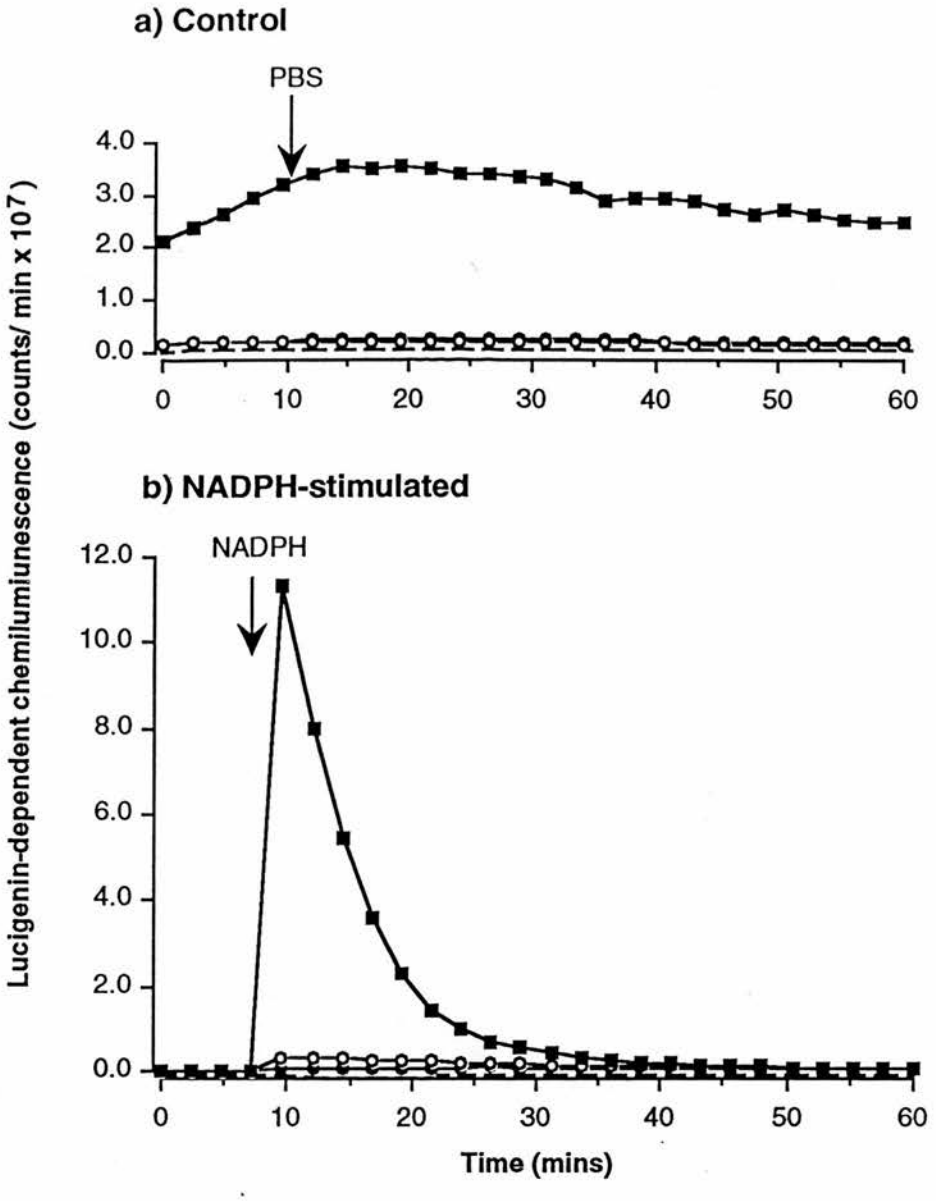

Figure 9.10 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by mouse epididymal spermatozoa. a) Control chemiluminescence; b) NADPH-stimulated chemiluminescence. Experiments were carried out in triplicate, and the traces are representative examples.
Caput spermatozoa = —■—; corpus spermatozoa = —○— ;
cauda spermatozoa = —●— ; and BWW control = - - - - - .

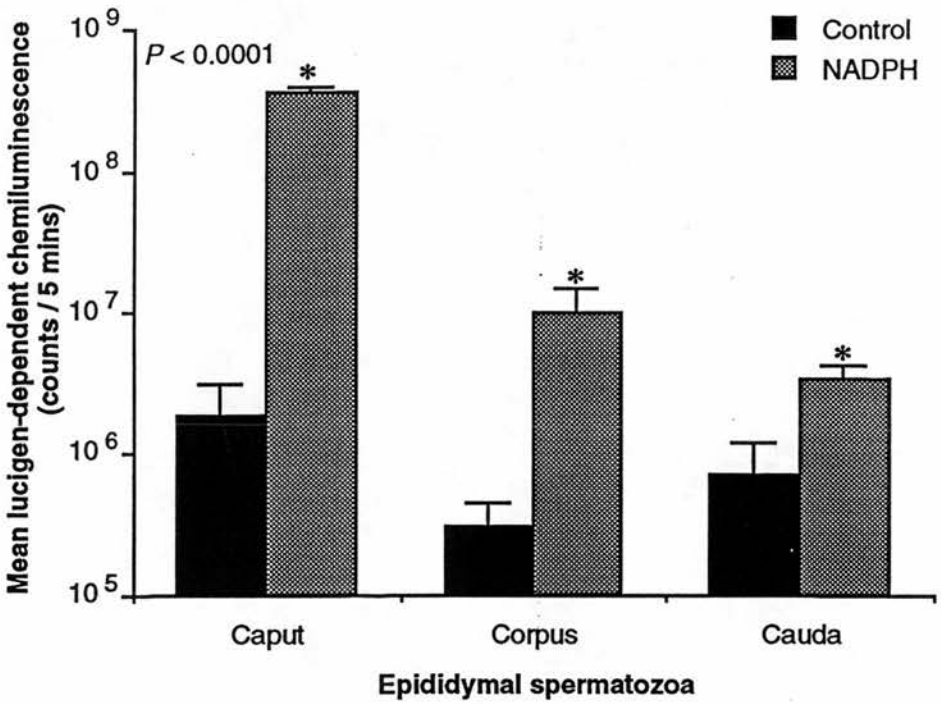

Figure 9.11 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by mouse epididymal spermatozoa. Results are the means ± S.E. of 3 separate experiments. The NADPH induced responses were significantly higher than control responses, $*P < 0.05$. Overall, the reponses were significantly different from one another ($P = 0.0001$). Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

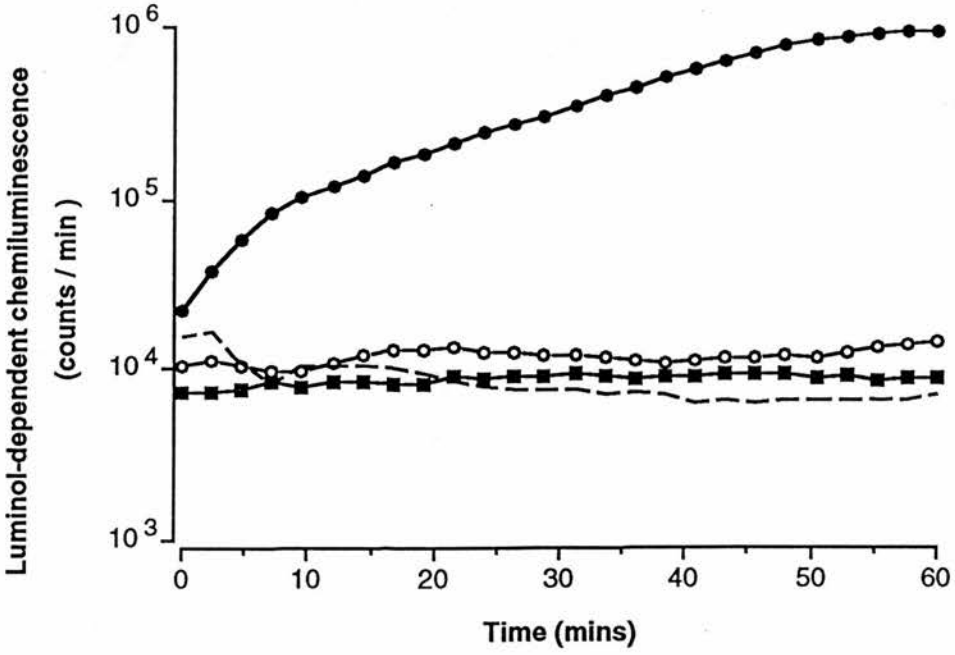

Figure 9.12 Spontaneous HRP-enhanced, luminol-dependent chemiluminescence by mouse epididymal spermatozoa. Experiments were carried out in triplicate, and the traces are representative examples. Caput =—■—; Corpus = —o—; Cauda = —●—; and BWW control = - - - - - .

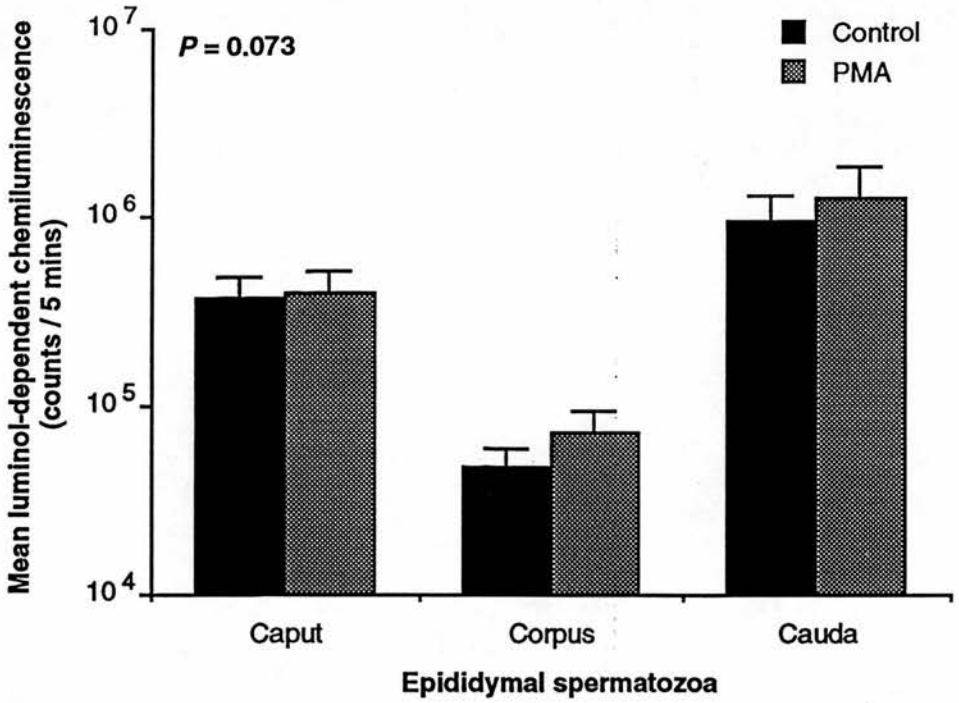

Figure 9.13 Effect of PMA (100nM) on luminol-dependent chemiluminescence by mouse epididymal spermatozoa. Results are the means ± S.E. of 3 separate experiments. There were no significant reponses to PMA. Overall, the responses were not significantly different from one another.

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

9.3.3 ROS generation by rat testicular germ cells.

Rat testicular germ cells did generate ROS, as monitored by both lucigenin and HRP-enhanced, luminol-dependent chemiluminescence, although the levels of the activity varied with respect to the maturational status of the cells under investigation, and with the conditions under which it was monitored.

Spontaneous ROS generation

Rat germ cells appeared to spontaneously generate only very low levels of the superoxide anion, as indicated by lucigenin-dependent chemiluminescence (Figures 9.18a and 9.19). The extent to which the different stages of germ cell exhibited this ability did not significantly differ from one another.

Monitoring hydrogen peroxide generation with HRP-enhanced, luminol-dependent chemiluminescence, revealed that rat testicular germ cells generated this molecule spontaneously, without need for prior stimulus (Figure 9.20). The levels of spontaneous hydrogen peroxide generated by each of the stages of germ cell differentiation did not significantly differ from one another (Figure 9.21).

Exogenous NADPH-induced ROS generation

All of the different germ cell suspensions tested, generated increased levels of the superoxide anion, in response to exogenous NADPH, as monitored by lucigenin-dependent chemiluminescence (Figures 9.18b and 9.19), although the magnitude of the responses varied, dramatically, depending on the differential status of the germ cells. Pachytene spermatocytes generated the greatest levels of the superoxide anion, in response to exogenous NADPH, and round spermatids generated approximately twice the level of superoxide anion, in response to exogenous NADPH, as the elongate spermatids, although the elongate spermatids themselves generated significant levels of the superoxide

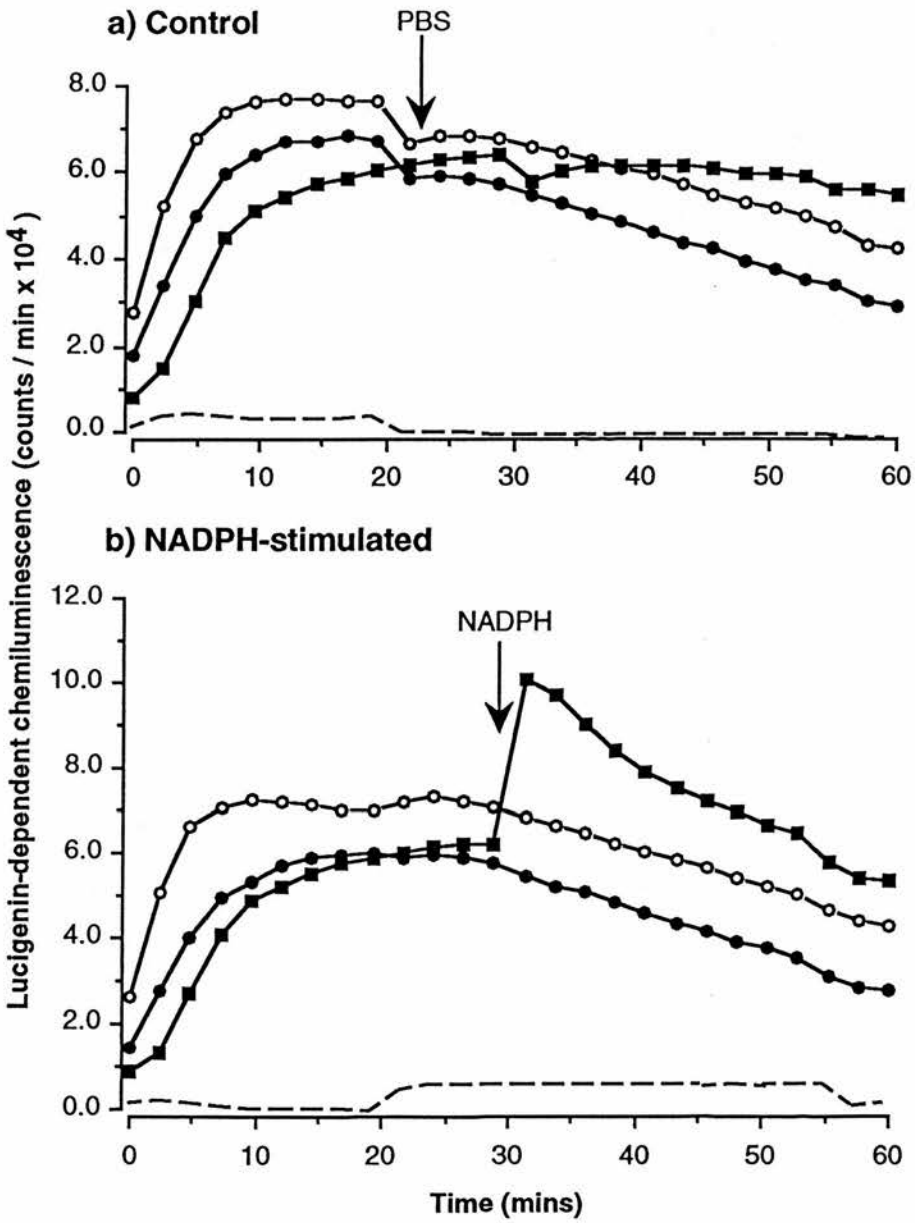

Figure 9.14 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by rat epididymal spermatozoa. a) Control chemiluminescence; b) NADPH stimulated chemiluminescence. Experiments were carried out in triplicate, and the traces are representative examples.
Caput spermatozoa = —■—; corpus spermatozoa = —o—;
cauda spermatozoa =—●— ; and BWW control = - - - - - .

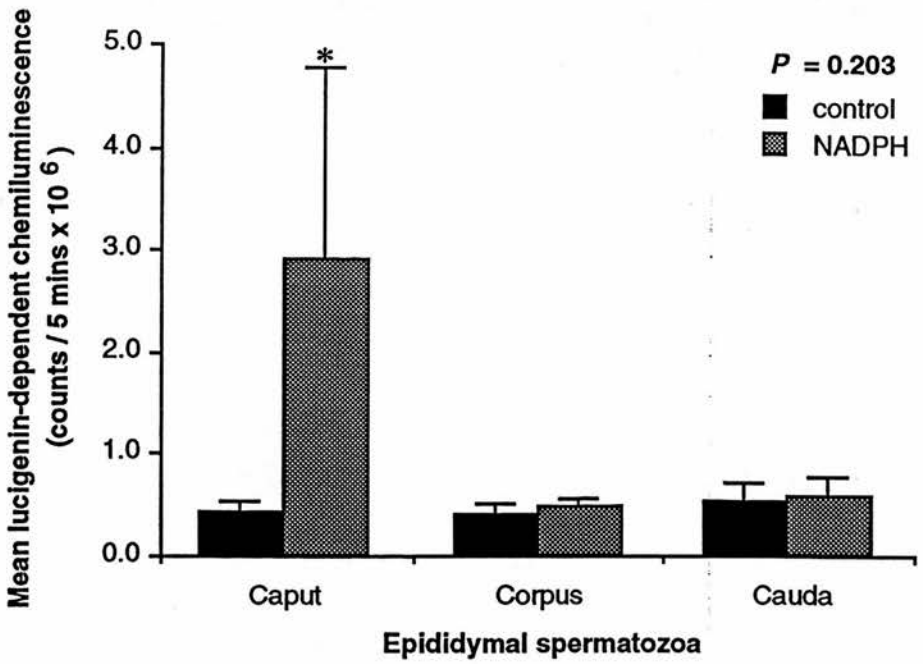

Figure 9.15 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by rat epididymal spermatozoa. Results are the means ± S.E. of 3 separate experiments. Only caput spermatozoa gave a responseto NADPH significantly greater than the control reponse, $*P < 0.05$. Overall, the responses were not significantly different from one another ($P = 0.203$).

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

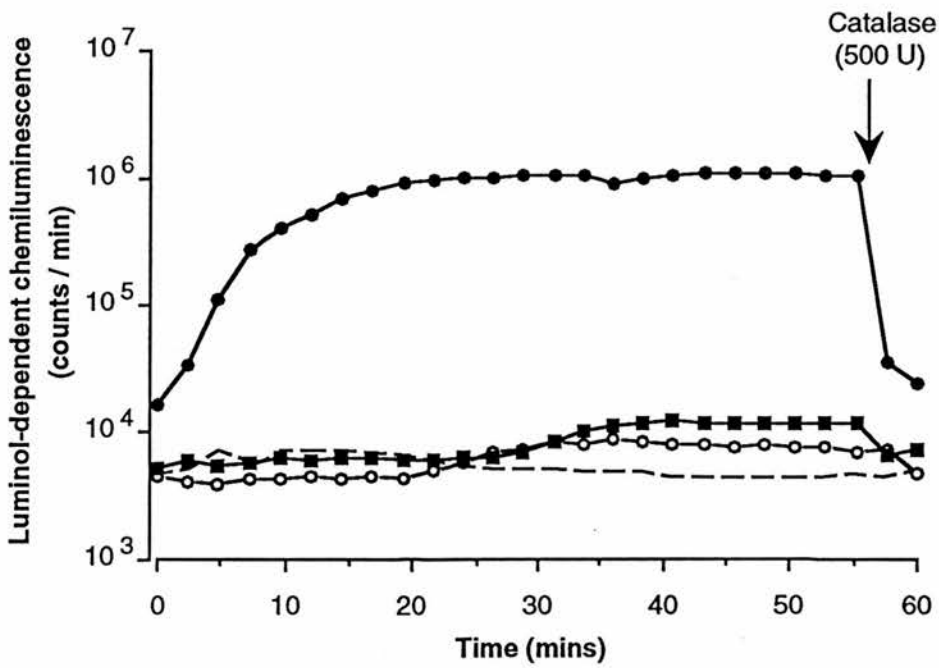

Figure 9.16 Spontaneous HRP-enhanced, luminol-dependent chemiluminescence by rat epididymal spermatozoa. Catalase (500U) was added to some suspensions, near the end of the run, to confirm the identity of the ROS being measured. Experiments were carried out in triplicate, and the traces are representative examples.
Caput = —■—; Corpus = —o— ; Cauda =—●— ;
and BWW control = - - - - - .

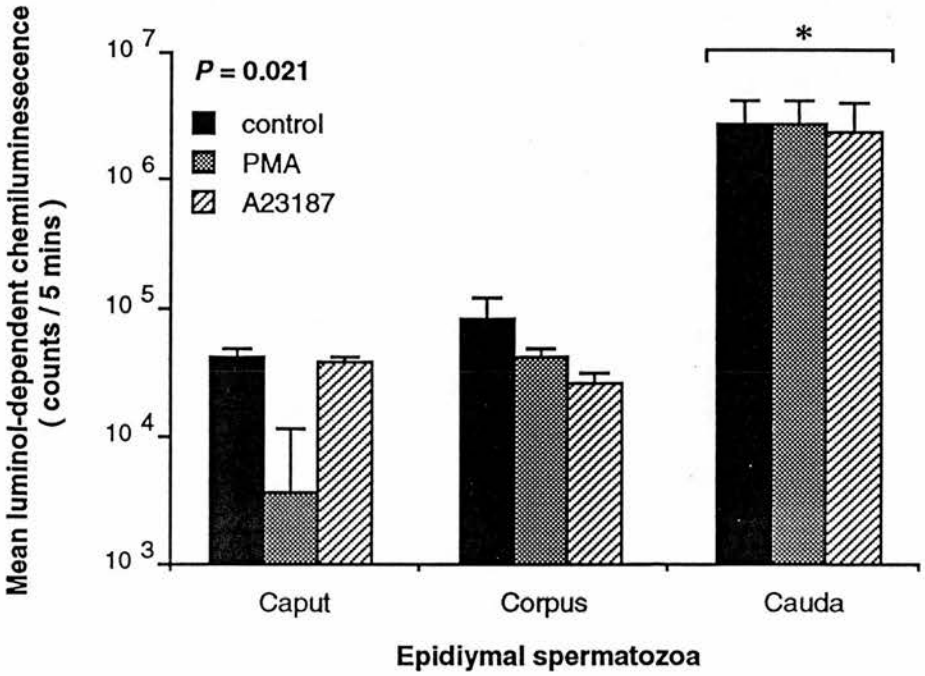

Figure 9.17 Effect of PMA (100nM) and A23187 (2.5μM) on luminol-dependent chemiluminescence by rat epididymal spermatozoa. Results are the means ± S.E. of 3 separate experiments. There were no significant reponses to PMA or A23187, but luminol-dependent chemiluminescence by cauda spermatozoa was significantly higher than that by spermatozoa from the other regions of the epididymis, $*P < 0.05$. Overall, the results were significantly different from one another ($P = 0.021$).

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

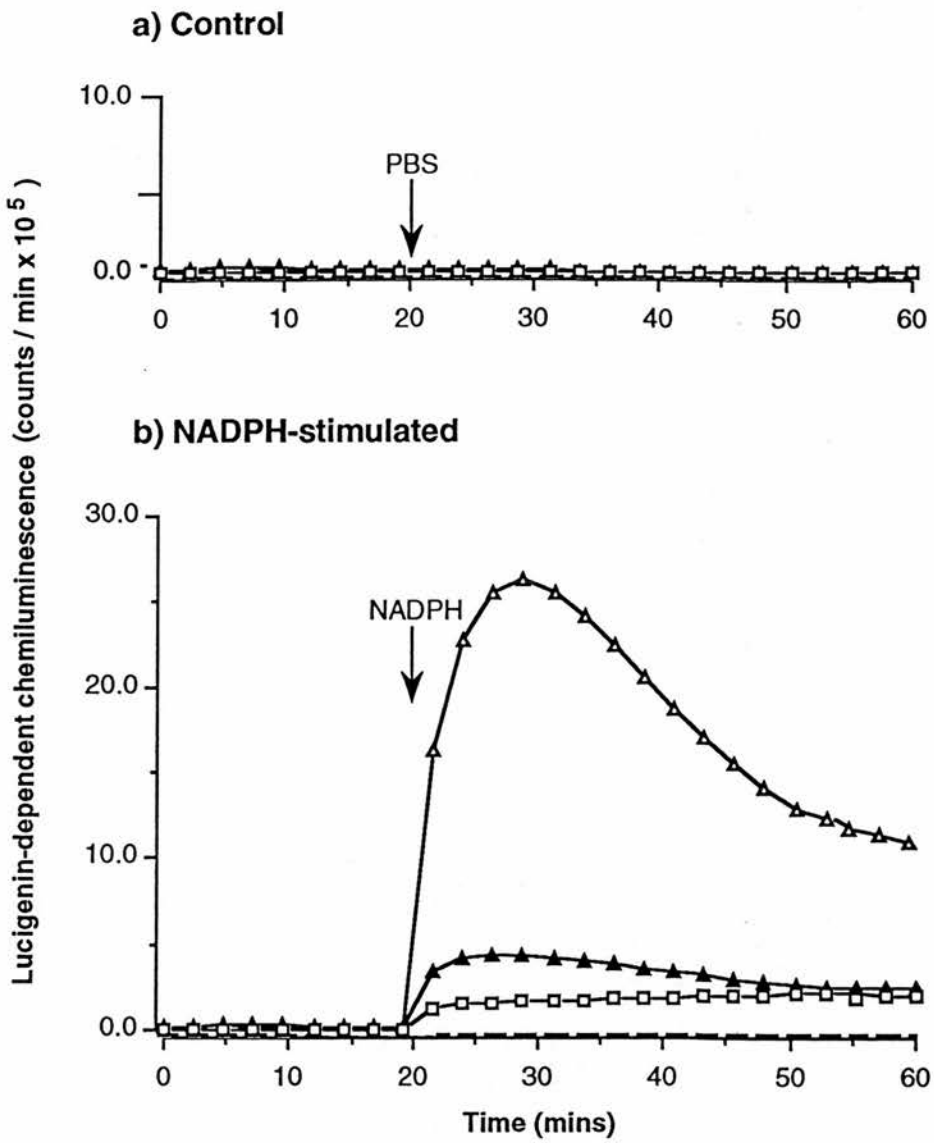

Figure 9.18 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by rat germ cells a) Control chemiluminescence; b) NADPH stimulated chemiluminescence. Experiments were carried out in triplicate, and the traces are representative examples.
Pachytene spermatocytes = —△— ; round spermatids = —▲— ; and elongate spermatids = —□— ; and BWW control = - - - - - .

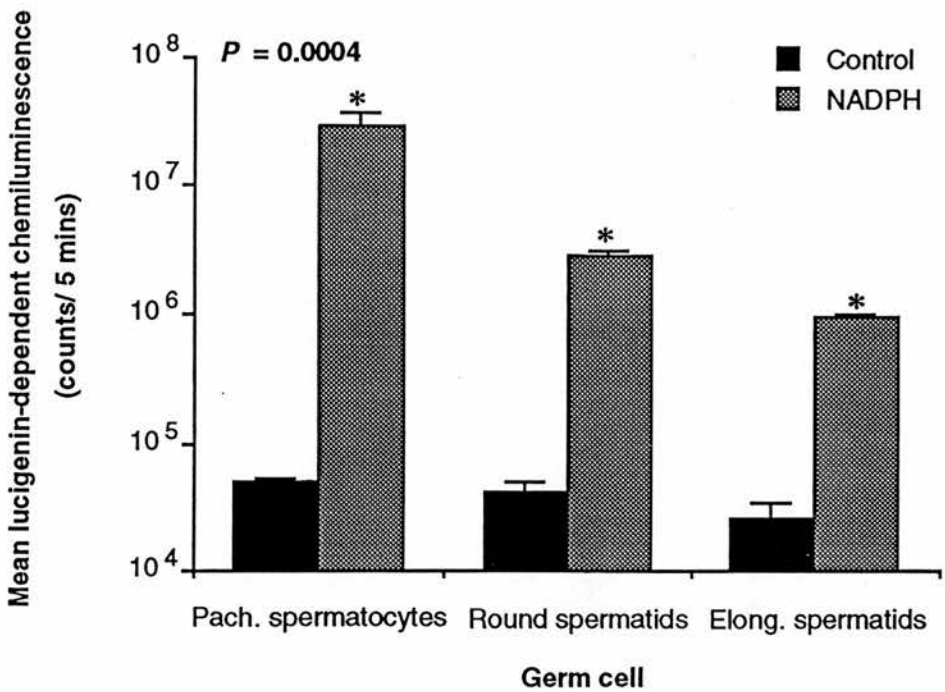

Figure 9.19 Effect of NADPH (500μM) on lucigenin-dependent chemiluminescence by rat germ cells. Results are the means ± S.E. of 3 separate experiments. The NADPH-induced responses were significantly higher than control responses, $*P < 0.05$. Overall, the responses were significantly different from one another ($P = 0.0004$).

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

anion in response to exogenous NADPH, as compared to spontaneous superoxide generation (Figure 9.19).

PMA and A23187-induced ROS generation

None of the germ cell preparations showed increased levels of HRP-enhanced, luminol-dependent chemiluminescence in response to addition of PMA or the divalent cation ionophore, A23187 (Figure 9.21), indicating that these reagents do not stimulate ROS generation by the rat testicular germ cell suspensions being studied here.

9.4 Discussion

The aims of the work described in this chapter were to determine whether the spermatozoa of common laboratory species generated reactive oxygen species (ROS), and to follow the ontogeny of this activity during epididymal maturation and spermiogenesis. The results presented in this chapter indicate that the epididymal spermatozoa of species such as the guinea pig, rat, mouse, and hamster generate, and release, ROS. These results emphasise the ubiquitous nature of ROS generation by mammalian spermatozoa, reinforcing results previously obtained in such species as the golden hamster (Bize and Sharpe, 1990), the mouse (Alvarez and Storey, 1984), and the rat (Kumar *et al*, 1990). However, these are the first data to indicate that guinea pig spermatozoa are competent to generate ROS. ROS generation was monitored by chemiluminescence techniques, and was shown to be modulated by the addition of NADPH.

The spermatozoa used in the above experiments showed physical characteristics typical of epididymal spermatozoa. One way in which these typical characteristics were manifested was that the spermatozoa showed increasing motility with increasing epididymal maturity. Acquisition of sperm motility during epididymal maturation is one of the most prominent

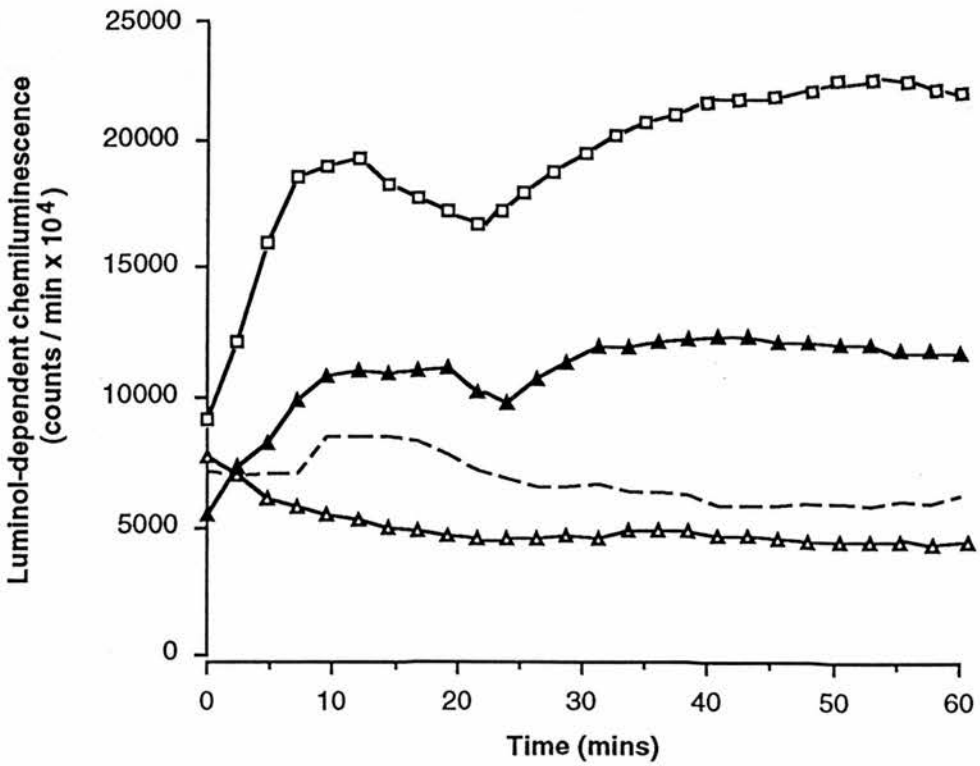

Figure 9.20 Spontaneous HRP-enhanced, luminol-dependent chemiluminescence by rat germ cells. Experiments were carried out in triplicate, and the traces are representative examples.
Pachytene spermatocytes = —△— ; round spermatids = —▲— ; and elongate spermatids = —□— ; and BWW control = - - - - - .

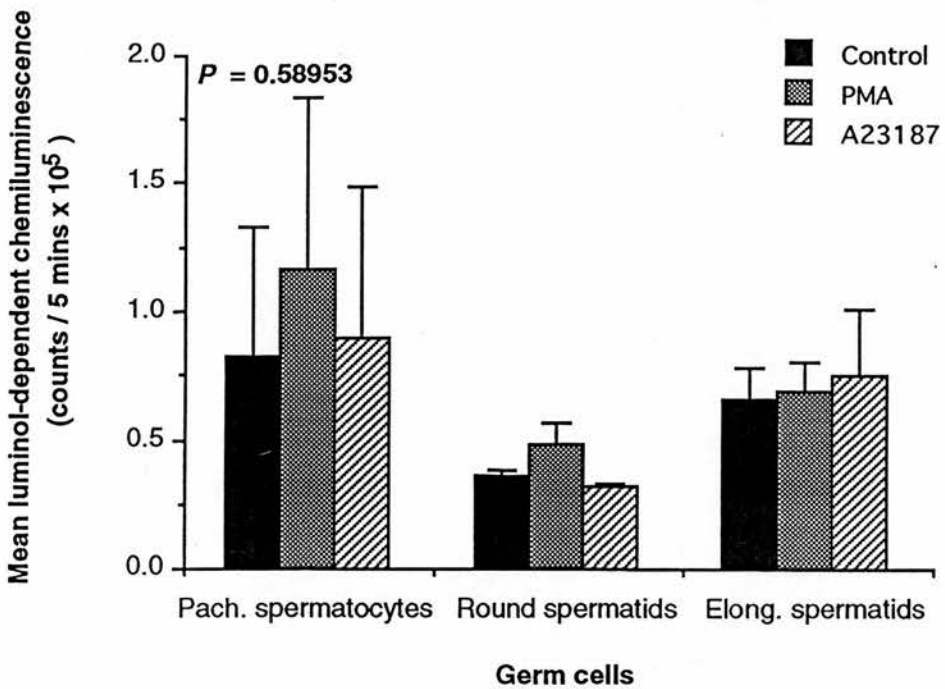

Figure 9.21 Effect of PMA (100nM) and A23187 (2.5μM) on luminol-dependent chemiluminescence by rat testicular germ cells. Results are the means ± S.E. of 3 separate experiments. There were no significant reponses to PMA or A23187, and overall, the levels of chemiluminescence by the different cell types were not significantly different from one another.

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

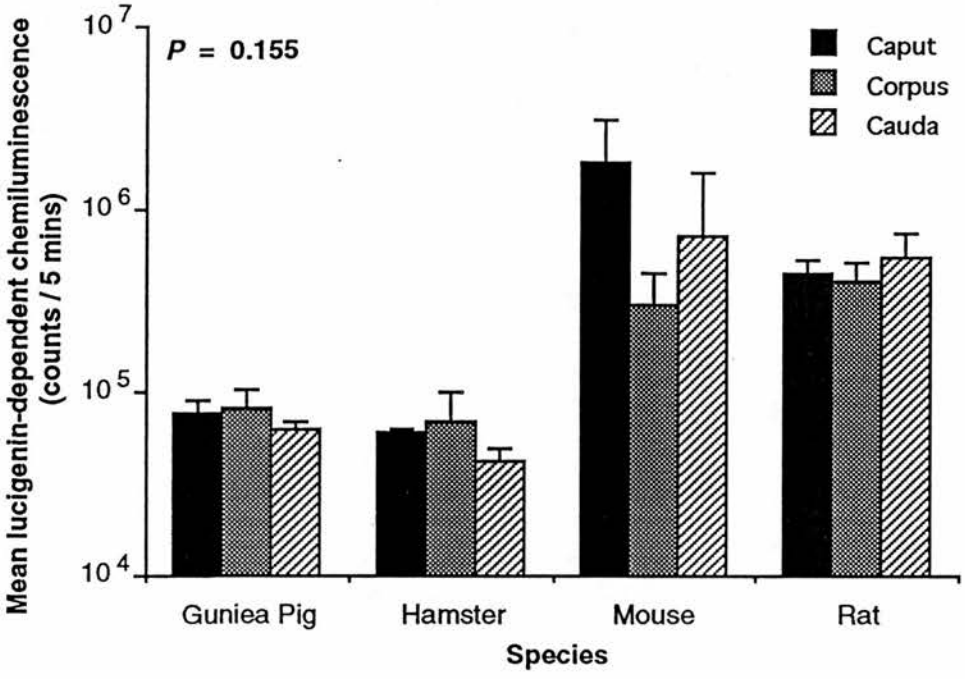

Figure 9.22 Spontaneous lucigenin-dependent chemiluminescence by the epididymal spermatozoa of various mammalian species. Results are the means ± S.E. of 3 separate experiments. Spontaneous superoxide anion generation did not significantly differ between the species.

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

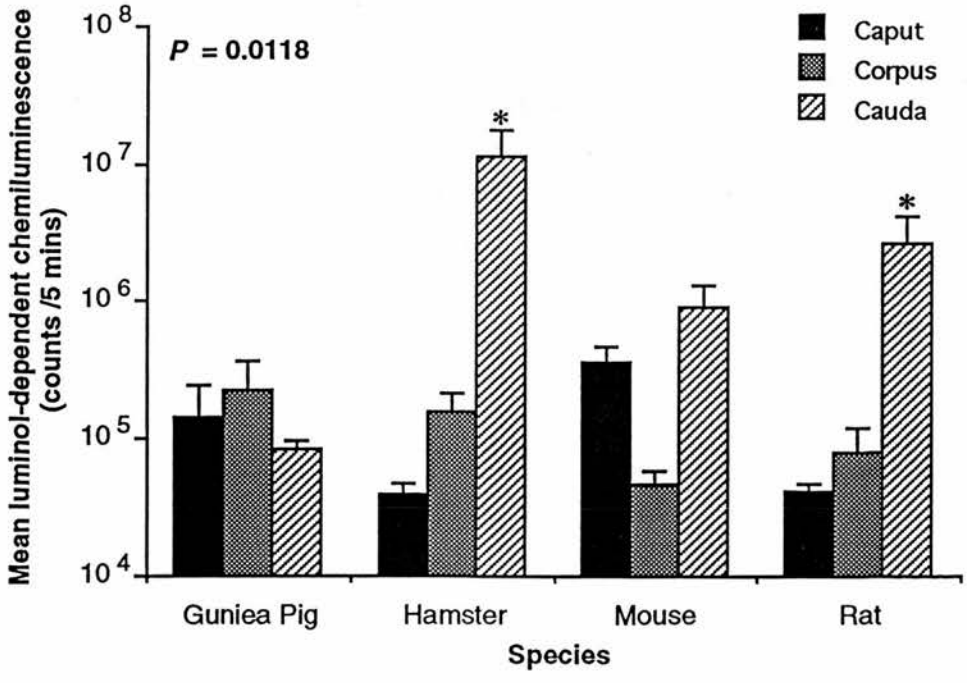

Figure 9.23 Spontaneous HRP-enhanced, luminol-dependent chemiluminescence by the epididymal spermatozoa of various mammalian species. Results are the means ± S.E. of 3 separate experiments. Spontaneous HRP-enhanced, luminol-dependent chemiluminescence by hamster and rat cauda spermatozoa was significantly higher than that by spermatozoa from other regions of the epididymis, $*P < 0.05$. Overall, the levels of spontaneous ROS generation observed, did significantly differ from one another ($P = 0.0118$).

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

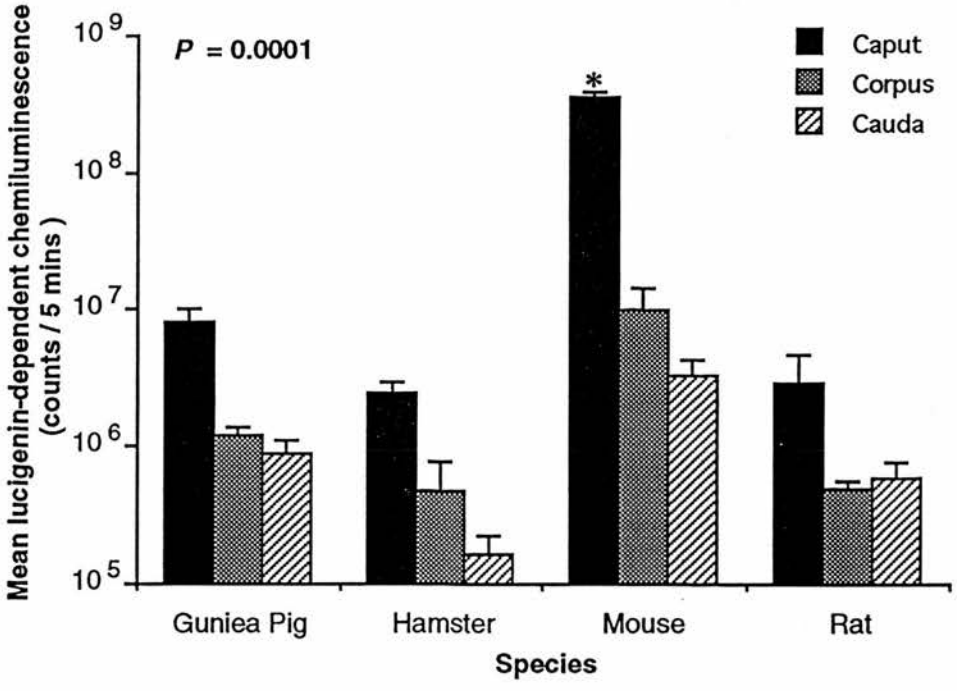

Figure 9.24 Comparison of NADPH-induced superoxide anion generation by the epididymal spermatozoa of various mammalian species, as monitored by lucigenin-dependent chemiluminescence. Results are the means ± S.E. of 3 separate experiments. Only the caput spermatozoa of the mouse generated levels of the superoxide anion, in response to NADPH, that were significantly different from all the the other cell responses, $*P < 0.05$. However, overall, the responses were significantly different fromone another ($P = 0.0001$).

Analysis of variance (ANOVA) was carried out in order to determine the statistical significance of the results obtained.

features associated with this process, and has been extensively studied (e.g. Hoskins *et al,* 1975; Vijayaraghavan and Hoskins, 1990; Moore *et al,* 1992; review, Mori and Ishijima, 1989). It is thought that the development of motility is due to changes in the sperm plasma membrane, and it is dependent on ATP, cAMP, and magnesium (Mori and Ishijima 1989). Another factor thought to influence the acquisition of sperm motility during epididymal maturation is thiol status. During epididymal transit thiols, present on the sperm surface, become cross-linked, forming stabilizing disulphide bonds (Calvin and Bedford, 1971; Chang and Zirkin, 1978; Yeung *et al,* 1994). Hydrogen peroxide will oxidize thiols, forming disulphide bonds (Halliwell and Gutteridge, 1986), so it is possible that the increased levels of hydrogen peroxide generated by corpus and cauda spermatozoa are responsible for the oxidation, and hence cross-linking of thiols, thus making the increase in hydrogen peroxide generation a causative factor in the acquisition of sperm motility. One way to test this hypothesis would be to add hydrogen peroxide to caput spermatozoa, and observe whether there were any changes in the motility of the cells. If the cells became motile, without any concomitant reduction in their overall functional integrity, then this strategy could possibly be developed as a therapeutic treatment for sperm dysfunction, characterized by a lack of motility.

Another phenomenon of epididymal maturation of spermatozoa is the migration and loss of the cytoplasmic droplet (Cortadellas and Durfort, 1994). This was shown many years ago to be a feature of the epididymal maturation of the spermatozoon (Branton and Salisbury, 1947), and it was a distinct feature of the spermatozoa used in these experiments, i.e. a significant reduction in the presence of the cytoplasmic droplet as epididymal maturation ensued. The increasing prevalence of the cytoplasmic droplet as one goes backward through the epididymis, may be one explanation for the concomitant increase in the cells response to NADPH. It is possible that enzymes present in the cytoplasmic droplet contribute to ROS generation, and

that the enzyme system responsible for ROS generation, in response to the administration of exogenous NADPH, is cytoplasmic in location. However, this does not explain the same results in guinea pig spermatozoa, as spermatozoa from all regions of the guinea pig epididymis, failed to show the presence of the cytoplasmic droplet, the reason for this being undetermined.

On the whole, the patterns of ROS generation, as monitored by luminol- and lucigenin-dependent chemiluminescence, by the spermatozoa of the different animals were very similar, especially with respect to their responses to exogenous stimuli, but did vary, to some extent, with regard to spontaneous, chemiluminescence-monitored, ROS generation. Dealing firstly with spontaneous ROS generation, this phenomenon was quite uniformly expressed across the species (Figures 9.22 and 9.23), with the levels of superoxide anion generation, as indicated by lucigenin-dependent chemiluminescence, remaining fairly constant throughout epididymal maturation. The mouse and rat showed much higher lucigenin-dependent chemiluminescent signals than the spermatozoa of the guinea pig or hamster, the latter two species generating very similar intensities of chemiluminescent signals. The consistent nature of, apparent, spontaneous superoxide anion generation by epididymal spermatozoa, during epididymal maturation, observed in this study contrasts with the results presented by Kumar *et al* (1990). Their work showed that the epididymal spermatozoa of the rat showed a marked increase in spontaneous superoxide anion generation as they underwent maturation in the epididymis. The conflicting nature of the two sets of results could possibly be explained by the fact that different techniques were used to monitor superoxide anion generation in the two studies. The Kumar study monitored superoxide anion generation by a spin-trapping method, the results from which are notoriously difficult to analyse, and which is possibly not comparable to the chemiluminescent techniques used here for the determination of superoxide anion generation.

It is probable that the profile of superoxide anion generation by epididymal spermatozoa is profoundly influenced by the underlying activity of the superoxide scavenging enzyme, SOD (Perry *et al,* 1993; Kumar *et al,* 1990). Thus, it is possible that the levels of the superoxide anion generated by epididymal spermatozoa do increase as the spermatozoa undergo epididymal maturation, but that this change is masked by a compensatory increase in SOD activity. Some evidence for this hypothesis was to be found in the increased level of spontaneous hydrogen peroxide generation observed in caudal epididymal spermatozoa. Thus, in all species examined, epididymal maturation was accompanied by increased levels of hydrogen peroxide generation. Moreover, as caudal epididymal spermatozoa underwent incubation *in vitro,* they showed a marked enhancement of hydrogen peroxide production, that was not exhibited by caput epididymal spermatozoa. In the light of data indicating that hydrogen peroxide generation is an essential element in the cascade of events leading to the acrosome reaction in hamster spermatozoa (Bize and Sharpe, 1990; Bize *et al,* 1991), it is possible that the generation of this oxidant is an important, ubiquitous feature of sperm capacitation.

SOD is clearly an important enzyme in terms of epididymal maturation, and it is probably significant that the levels of its expression in the epididymis vary, dependent upon the region of the epididymis (Perry *et al,* 1993; Kumar *et al,* 1990). It has been shown that the cauda epididymis contains the highest level of SOD, with a gradual decrease as one goes backwards through the epididymis (Perry *et al,* 1993). This would be in line with the principal role of the caudal epididymis, i.e. as a storage organ (Amann *et al,* 1993). It is possible that in the cauda epididymis, spermatozoa are particularly vulnerable to oxidative stress, possibly due to increased generation of ROS by the spermatozoa themselves in this location, and hence elevated levels of SOD are required here, in order to protect the spermatozoa from undergoing

peroxidative damage, before they are ejaculated into the protective, antioxidant environment of seminal plasma (Jones *et al,* 1979; Zini *et al,* 1993). It has also been postulated that some SOD is adsorbed onto the plasma membrane of the spermatozoon (Holland *et al,* 1982; Lasso *et al,* 1994). This membrane-associated SOD probably then catalyzes the dismutation of spontaneously generated superoxide, to hydrogen peroxide and oxygen. Since the hydrogen peroxide generated in the reaction would be detrimental to the spermatozoa, it might be anticipated that changes in SOD activity might be accompanied by alterations in peroxide metabolizing enzymes such as glutathione peroxidase and glutathione transferase, in the epididymis. This is indeed the case, i.e. it has been shown that the cauda epididymis exhibits the highest levels of activities of these two enzymes, compared to the other regions of the epididymis (Perry *et al,* 1993; Veri *et al,* 1993; Veri *et al,* 1994).

A further possibility regarding the variation in the levels of ROS by epididymal spermatozoa is that it is a result of an increase in the metabolism of the spermatozoa, primarily increases in mitochondrial enzyme activity and the concomitant development of motility (Garbers *et al,* 1973). As mentioned in a previous chapter of this thesis (Chapter 4), electron leakage from the mitochondrial electron transport chain may contribute to observed levels of ROS generation by cells (Nohl and Hegner, 1978) and it has been shown that in rabbit spermatozoa, ROS generation is a direct consequence of mitochondrial electron leakage effecting the partial reduction of molecular oxygen to superoxide anion, which subsequently dismutates, under the action of mitochondrial SOD, to hydrogen peroxide (Boveris and Chance, 1973; Holland *et al,* 1982). To test whether mitochondrial activity was in any way contributing to the observed increase in ROS generation, the effect of mitochondrial inhibitors on ROS generation by spermatozoa from the various regions of the epididymis could be assessed. Unfortunately, such experiments were beyond the scope of this preliminary study.

In order to determine whether the generation of ROS by the spermatozoa of laboratory species exhibits the same general features as the expression of this activity in human spermatozoa, the influence of NADPH, PMA and A23187, was investigated. Neither PMA or A23187 resulted in any significant increases in the generation of hydrogen peroxide by any of the spermatozoa tested, as monitored by luminol-dependent chemiluminescence, indicating that elevation of intracellular calcium levels or stimulation of PKC, does not appear to have any effect on ROS generation by these species. This appears to be in contrast with the situation in human spermatozoa, since these cells have been shown to generate hydrogen peroxide in response to A23187 and PMA (Aitken and Clarkson, 1987a and b). It may be that the generation of ROS by human spermatozoa in response to PMA and A23187 is a consequence of incomplete cytoplasmic extrusion and that this phenomenon is not observed in common laboratory species that do not normally exhibit the retention of excess cytoplasm. The reason for the lack of an observed response to A23187 and PMA by rat testicular germ cells, cells which still contain reasonable amounts of cytoplasm, remains, at present, uncertain, and will not be addressed any further at this juncture.

In agreement with the work carried out on human spermatozoa, the epididymal spermatozoa of the 4 mammalian species investigated here, generated the superoxide anion in response to exogenous NADPH, as shown by an increase in lucigenin-dependent chemiluminescence. In all species, the response to NADPH followed a consistent trend, with the response being negatively correlated with the epididymal maturity of the spermatozoa. This pattern of response was observed in all species, the epididymal spermatozoa of the rat being an extreme example of this variation, since the cauda and corpus spermatozoa did not generate any extra superoxide anion in response to NADPH, whiist the caput spermatozoa were active in this regard.

Their are various possible explanations for the variable responses to NADPH given by epididymal spermatozoa. One possibility is based upon the antioxidant capacities of the spermatozoa. It has already been suggested that epididymal differences in the SOD content of the spermatozoa may have profound effects on spontaneous ROS generation by epididymal spermatozoa, and this may also be true of NADPH-induced superoxide anion generation. Total superoxide generation by the cauda spermatozoa may not be detected by lucigenin-dependent chemiluminescence due to the SOD present, scavenging some or all, in the case of the rat, of the superoxide generated.

Cell permeability to NADPH could also be a factor regulating the response to NADPH, since the fluidity of the sperm plasma membrane is known to change during epididymal maturation (Hall *et al,* 1991; Rana *et al,* 1993). A decrease in permeability, as the spermatozoa undergo epididymal maturation, would possibly result in a decrease in the NADPH response. However, it has been shown that spermatozoa actually become more permeable to small molecules as they undergo maturation in the epididymis (Schlegel *et al,* 1986). Whether these changes in permeability would apply to NADPH or not, is not certain, and it may be the case that permeability to NADPH is differentially altered/regulated. To address this issue, experiments could be performed using permeabilized, or detergent solubilized epididymal spermatozoa. A third possibility is that the formation of stabilizing disulphide bonds, during sperm epididymal maturation, results in a decrease in permeability to NADPH, the stabilization brought about by these changes being involved with the acquisition of sperm motility and nuclear stability (Calvin and Bedford, 1971; Chang and Zirkin, 1978; Hino *et al,* 1986).

A further explanation for the gradual decrease in superoxide anion generation in response to NADPH is that the activity of the enzyme system(s) responsible for NADPH-induced superoxide anion generation undergo maturational modification in the epididymis, resulting in a down-regulation of

activity. The activities of numerous sperm enzymes change during epididymal maturation and spermiogenesis (Hoskins *et al,* 1975; Chang and Zirkin, 1978; Kumar *et al,* 1990; Peltola *et al,* 1992; Rigaudière *et al,* 1992; Perry *et al,* 1993; Saunders *et al,* 1993; Tulsiani *et al,* 1993), and it is possible that NADPH oxidase activity is similarly differentiation/maturation dependent.

Leaving to one side the elucidation of the biochemical mechanisms responsible for ROS generation by epididymal spermatozoa and post-meiotic, testicular germ cells, the work presented in this chapter has emphasized the potential importance of oxidant : antioxidant balance during the differentiation and maturation of mammalian spermatozoa. ROS generation appears to be a ubiquitous feature of mammalian male germ cells, and the results presented here emphasize the importance of the defensive strategies that must exist in order to protect these cells from oxidative stress. Clearly, normal testicular and epididymal function must depend upon the maintenance of a delicate balance of factors promoting and inhibiting, oxidative processes within the testes, epididymis and germ cells of the mammalian male.

9.5 Summary and conclusions

The work described in this chapter demonstrates that ROS generation by mammalian epididymal spermatozoa is a conserved feature of these cells, that varies with respect to the maturational status of the cells. It has also been shown, in the rat, that the ability to generate ROS is expressed by testicular germ cells as well as in fully differentiated spermatozoa. The biochemical mechanisms by which male germ cells generate ROS have not been identified by these studies, although some similarities, and differences with ROS generation by ejaculated human spermatozoa have been shown.

The realization that ROS generation is a conserved feature of mammalian spermatozoa, and possibly germ cells, will enable much more in-depth studies to be undertaken into the function(s) and control of ROS generation by these

cells in animal models. However, to be of any significance, such studies would have to be carried out hand in hand with studies into antioxidant levels in the immediate, external, environment of the cells, and into their own antioxidant capacities. Eventually, it should be possible to manipulate the oxidative balance in the testis and epididymis to reveal the roles of ROS in these organs, and to develop strategies with which to correct imbalances in the oxidative status of the cells external milieu, and possibly that in the cells themselves.

Thus, the work described in this chapter has shown that it should be possible to develop an animal model to study ROS generation by spermatozoa and precursor germ cells, and the development of such a model will hopefully aid our understanding of the significance of redox mechanisms in the genesis, and maturation of mammalian spermatozoa.

Chapter 10

General discussion and conclusions

10.1 ROS generation by human spermatozoa

The work described in this thesis, has provided experimental evidence showing that human spermatozoa generate and release the superoxide anion via a NADPH oxidase-like system. However, evidence is also presented showing that although the NADPH oxidase systems of human spermatozoa and phagocytic leucocytes share some functional properties, they are not structurally, or even functionally the same. The experiments described in this thesis indicate that, as in leucocytes, ROS generation by human spermatozoa is enzymatic in nature, and utilizes NADPH as electron donor, univalently reducing molecular oxygen to superoxide. Further shared characteristics include the probable involvement of a flavoprotein, and a role for protein phosphorylation in ROS generation, at least when endogenous substrate is being employed. Thus it appears that the activity of the NADPH oxidase of human spermatozoa can, in some circumstances, be regulated by PKC-dependent protein phosphorylation, but it is not apparent if the protein phosphorylation subsequently induced, has the same effect in spermatozoa as it has in phagocytic leucocytes. In leucocytes, PKC appears to regulate NADPH oxidase assembly and activation (Wyman *et al*, 1987), whilst the sperm NADPH oxidase appears to require no prior assembly or activation for its activity, when supplied with exogenous NADPH.

Biochemical and molecular analyses of human spermatozoa have shown that the cellular components which form the NADPH oxidase of phagocytic leucocytes are probably not present in human spermatozoa. It does appear that like phagocytes, sperm ROS generation involves an NADPH oxidase system with a flavoprotein component. However, it was not possible to

demonstrate the existence of the characteristic low potential cytochrome b_{558} in this system, or any of the other cellular components associated with the NADPH oxidase of phagocytic leucocytes. Such results are in line with what has been recently shown in another, non-phagocytic, cell expressing NADPH oxidase-like activity. It has now been shown that the fibroblast NADPH oxidase system is distinct from that of leucocytes (Meier *et al*, 1993; Emmendorffer *et al*, 1993), and it has been demonstrated that fibroblasts do not express molecules that are identical to the component parts of the leucocyte NADPH oxidase. Thus, it is possible that the situation is similar in human spermatozoa, i.e. although human spermatozoa possess a ROS generating system that appears superficially similar to the leucocyte NADPH oxidase, the two systems are actually structurally and genetically distinct.

However, further investigations are clearly warranted, to categorically confirm that components of the leucocyte NADPH oxidase are not present in human spermatozoa. Such experiments could take advantage of recent advances in using molecular techniques to identify the presence of specific mRNA's in spermatozoa. Until quite recently, it was thought that spermatozoa were transcriptionally and translationally inactive, and did not contain any mRNA. However, although it has still not been shown that spermatozoa are transcriptionally active, the presence of residual mRNA molecules in human spermatozoa has been demonstrated (Miller *et al*, 1994). Thus, by extracting this residual mRNA, it should be possible to amplify and isolate mRNA species corresponding to NADPH oxidase components. This could be achieved by conducting reverse-transcriptase polymerase chain reaction (RT-PCR) analyses of extracted sperm mRNA, using primers based upon sequence data pertaining to the components of the NADPH oxidase. Such a technique would be extremely sensitive and, should detect the relevant molecules if they are present even at very low levels.

As known leucocyte NADPH oxidase components could not be detected in human spermatozoa using the methods available, techniques were developed to identify novel sperm proteins involved in ROS generation by these cells. Utilizing such techniques numerous components of human spermatozoa were identified capable of ROS generation, when supplied with NADPH, possibly indicating the existence of various isoforms of the NADPH oxidase-like system, or indicating that human spermatozoa possess more than one cellular system with the potential for ROS generation. The results described in this thesis raise the possibility that human spermatozoa possess molecules, e.g. LDH C_4, diaphorase, and NOS, which have the potential for ROS generation, as well an NADPH oxidase-like system, specifically designed for superoxide anion generation. In order to identify a component of the sperm NADPH oxidase-like system, a 55kDa protein, designated $sp55^{sox}$, apparently involved in ROS generation by human spermatozoa, was isolated and a polyclonal antibody raised against it. The antibody was subsequently used to investigate the cellular distribution of $sp55^{sox}$, to determine its role in NADPH-induced superoxide anion generation by human spermatozoa, and latterly, to screen a human testicular cDNA library cloned into the expression vector λgt11.

The partial cDNA for the gene encoding an antigen recognized by the anti-$sp55^{sox}$ IgG, was isolated from a human testicular cDNA expression library. The insert was approximately 1.9kb in length and was partially sequenced. The sequence data from the 5' -end showed very strong homology to the triple-helical domain of the alpha 1 sub-unit of human collagen, type VI, although the 3' -end did not show any significant homology with collagen VI, or any other published nucleotide sequence. Western blot analyses revealed that the $sp55^{sox}$ antigen possessed antigens recognised by a polyclonal antibody raised against human collagen, type VI, and similarly the anti-$sp55^{sox}$ IgG recognized epitopes present in the alpha 1 and 2 sub-units of

human collagen, type VI, indicating that the molecules shared similar structural domains, and confirming the homology shown by sequence analyses. Thus, it appears that human spermatozoa contain a molecule. with a collagen like domain, $sp55^{sox}$, that is associated with NADPH-induced ROS generation by these cells.

How could such a molecule be associated with ROS generation? One role attributed to molecules with collagen-like domains is membrane anchoring (Hulmes, 1994), and thus $sp55^{sox}$ could be involved in anchoring the enzymatic component(s) of the sperm NADPH oxidase-like system to the plasma membrane of these cells. It is possible that the 80kDa protein present in human spermatozoa, identified during the 2'5'ADP affinity chromatography stages of the $sp55^{sox}$ purification protocol, is in fact the enzymatic component of the ROS generating system and that the 55kDa protein is merely involved in membrane anchoring, or plays some other regulatory role. Such a role could be the enhancement of ROS generation by human spermatozoa, in response to a physiological stimulus. A molecule with a collagen like domain, L-selectin, has been described as fulfilling such a role in the NADPH oxidase of phagocytic leucocytes (Waddell *et al*, 1994). In these cells certain stimuli of ROS generation induce the cross-linking of L-selectin molecules on the surface of the phagocyte, which subsequently triggers a rise in intracellular calcium and a concomitant enhancement/potentiation of ROS generation. Such a situation is only observed when physiological stimuli of ROS generation are involved and not when non-physiological stimuli such as PMA are used. It is possible that such a situation exists in human sperm. In this instance, the physiological stimulus could be a component or components, of the female reproductive tract. It has been shown that low molecular weight components of follicular fluid induce enhanced ROS generation by human spermatozoa, and capacitation (de Lamirande and Gagnon, 1995). It has also been shown that interaction of spermatozoa with the epithelial cells of the females

reproductive tract results in capacitation of spermatozoa (Kervancioglu *et al*, 1994). Thus, as a growing number of reports associate the capacitation process with ROS generation by spermatozoa, it is tempting to speculate that temporary adhesion of spermatozoa to epithelial cells, or the interaction of spermatozoa with follicular fluid or other female reproductive tract components, results in cross linking of the $sp55^{sox}$ molecules on the surface of the spermatozoon, which then somehow triggers enhanced ROS generation, and subsequent capacitation.

All of this is mere speculation and to address the true role of $sp55^{sox}$ in human sperm ROS generation the entire nucleic acid, and amino acid, sequence of the molecule will have to be deduced and then studies investigating its functional significance carried out. Such studies could include looking at the effect of the antibody on the interaction of spermatozoa with other cells, and artificially inducing the cross-linking of the molecule on the surface of the spermatozoon and monitoring concomitant ROS generation and sperm capacitation. It would also be interesting to fully characterize the 80kDa protein identified during 2'5'ADP affinity chromatography separation of sperm proteins. This may reveal that the 80kDa protein is the enzymatic portion of the system, directly responsible for ROS generation.

Thus, *in vivo,* ROS generation by human spermatozoa may be modulated by the interaction of the collagen-like $sp55^{sox}$, with the external environment of the spermatozoon, and the ensuing increased ROS generation may have physiological significance with regard to such events as capacitation and the acrosome reaction.

10.2 Biological and clinical considerations

The precise mechanism(s) by which ROS, such as superoxide and hydrogen peroxide, influence sperm function is currently unknown, although a few possibilities exist (Aitken and Fisher, 1994). However, it is now widely

appreciated that ROS-mediated infertility is a consequence of ROS-induced peroxidative damage to the plasma membrane of the cells, and it is now postulated that ROS generation has a physiological importance in such functional events as capacitation and the acrosome reaction (Bize and Sharpe, 1990; Bize *et al*, 1991; de Lamirande and Gagnon, 1993a, b and c; de Lamirande and Gagnon, 1995), and even possibly in sperm-zona interaction (Aitken *et al*, 1989; Aitken *et al*, 1995b).

The discovery that intact, human spermatozoa can be stimulated to generate ROS, through the addition of exogenous NADPH, may prove to be a very important and useful breakthrough, both biologically and clinically. This discovery may have profound consequences on the study of the involvement of ROS in normal sperm function, as described above, and for the study of ROS-mediated, male infertility and sub-fertility. Moreover, if human spermatozoa, from 'normal' donors, can be stimulated to generate ROS with NADPH, then this provides a useful way of recreating, in *vitro*, the controlled, redox modification of sperm components, and also the oxidative stress which the spermatozoa of some sub-fertile men experience *in vivo*. Thus, redox regulation of sperm function, along with the effect of peroxidative damage to the plasma membranes of spermatozoa, could easily be studied, without the need for complicated experimental procedures and equipment. With such an experimental tool available, it may then be possible to then go on and devise much more potent and useful therapeutic strategies with which to treat free radical damage-mediated male infertility and sub-fertility, than are available at the present time. It may also be possible to utilize such an *in vitro* system to deduce the physiological roles of ROS generation by spermatozoa, until such a time as the definitive physiological stimulus of such activity is identified.

10.3 Cross-species generation of ROS by human spermatozoa

The realization that ROS generation is a conserved feature of mammalian spermatozoa, and possibly germ cells, will enable much more in-depth studies to be undertaken into the function(s) and control of ROS generation by these cells in animal models. The work described in Chapter 9 of this thesis has shown that it should be possible to develop an animal model to study ROS generation by both spermatozoa and precursor germ cells, and the development of such a model will hopefully aid our understanding of the significance of redox mechanisms in the genesis, and maturation of mammalian spermatozoa.

The most useful animal with which to undertake such work would probably be the rat; as work already carried out in the human could possibly be exploited here. The anti-sp55^{sox} IgG described in this thesis, was used to probe the spermatozoa of various animal species, and of all the non-human spermatozoa investigated, the antibody only cross-reacted with the spermatozoa of the rat. The anti-sp55^{sox} IgG detected a 58kDa antigen in rat spermatozoa that was present throughout epididymal maturation, and which was, in the caput region, accompanied by an additional cross reactive protein with a molecular mass of 55kD. Immunofluorescent labelling studies carried out with the anti-sp55^{sox} IgG, revealed that antigen, recognised by the IgG, was located over the entire surface of the rat spermatozoon, although the antigen appeared to be concentrated over the acrosomal, mid-piece and principle tail regions of the spermatozoon. The expression of the antigen showed a marked decline as the spermatozoon underwent epididymal maturation and such results would correspond to those from the luminometry-based experiments, i.e. NADPH-induced ROS generation declined as epididymal maturation ensued. Thus, it is possible that the sperm components involved in ROS generation are modified during epididymal maturation, and

this would be a very interesting point from which to address the area of ROS generation by mammalian spermatozoa. Of course, such studies in the human would be almost impossible to carry out, and hence, the development of an animal model could seriously aid work and understanding in this field.

10.4 Summary and conclusions

In summary, the work described in this thesis has conclusively shown that human spermatozoa, and those of other mammalian species, possess at least one specialized mechanism for ROS generation. This mechanism, though sharing some functional properties of the NADPH oxidase of phagocytic leucocytes, is structurally distinct. It remains for the 'oxidase' of human spermatozoa to be conclusively identified, but hopefully the information supplied in this thesis will enable this in the very near future.

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