DX

189643

THE DEVELOPMENT OF MOTILITY IN SPERMATOZOA

CHRISTINE M.B. O'TOOLE

A thesis submitted in partial fulfilment of the requirements
of the University of North London for the
degree of Doctor of Philosophy

DECEMBER 1994

ACKNOWLEDGEMENTS

I would like to thank Drs. Carol Brownson and Pat Williams for their supervision, guidance, support, patience and without whose help this thesis would not have been made possible.

My gratitude also goes to all the technicians and post graduate students at both The City of London Polytechnic and The University of North London, especially Ranjit for his help with the oxygen electrode.

A special thank you goes to Glenn Freary for his unfailing sense of humour, which in turn kept me going.

Special mention needs to be given to Dr. Paul Bolwell, for his initial idea of my working with *Fucus serratus* and subsequent guidance and to Dr. Colin Brownlee, at The Marine Laboratory, Plymouth, for his advice on the measurement of intracellular calcium in the spermatozoa of *Fucus serratus*.

More recently, I would like to thank Professor Lynn Fraser and Susan Adeoya-Osiguwa for their support and patience whilst writing this thesis.

Finally, gratitude must also go to my family and friends for their endless understanding and good cheer.

ABSTRACT

Mammalian spermatozoa acquire the capacity for motility during passage through the epididymis. This study on rat spermatozoa shows that pH, cAMP and protein kinase C (PKC) all play an important role in the initiation of motility. pH has the most critical role and until the initial pH change in spermatozoa has occurred between the caput and caudal epididymal regions of the rat, second messengers are not effective in stimulating motility, but they are involved once such pH change has occurred.

The spermatozoa of *Fucus serratus* differ from mammalian spermatozoa in that they are released into the sea prior to fertilisation and the motility of these spermatozoa is initiated upon their release into sea water. The ionic composition of sea water plays an important role in this activation and it is evident that the presence of Na^+ is vital for the initiation of motility. This study shows that a Na^+/H^+ exchanger, a Na^+-dependent bicarbonate/chloride exchanger and a Na^+/K^+ pump, which regulate the concentration of Na^+, are present in *Fucus serratus* and integrated activity of these exchangers/pumps causes an increase in intracellular pH (pHi). An elevation in pHi correlates to an increase in motility, mediated through the activation of the dynein ATPase of the flagella. Motility and respiration of these spermatozoa are closely linked, probably because the ATP produced by respiration is used primarily by the dynein ATPase. Second messengers have also been implicated in the initiation/regulation of motility and respiration. Indirect evidence shows cAMP and PKC are present and regulate motility, possibly through the phosphorylation and thereby activation of key regulatory proteins, such as the Na^+/H^+ exchanger. A rise in intracellular Ca^{2+} is also associated with the activation of *Fucus serratus* spermatozoa but the exact mechanism by which such a rise regulates motility remains unclear.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 7

CHAPTER 1

Introduction

1.1. Fertilisation.

Fertilisation is the process whereby two sex cells (gametes) fuse together to create a new individual with genetic potential derived from both parents. Although the actual details of fertilisation vary enormously from species to species, the events of conception generally consist of four major activities:

I. Contact and recognition between sperm and egg.

II. Regulation of sperm entry into the egg.

III. Fusion of the genetic material of sperm and egg.

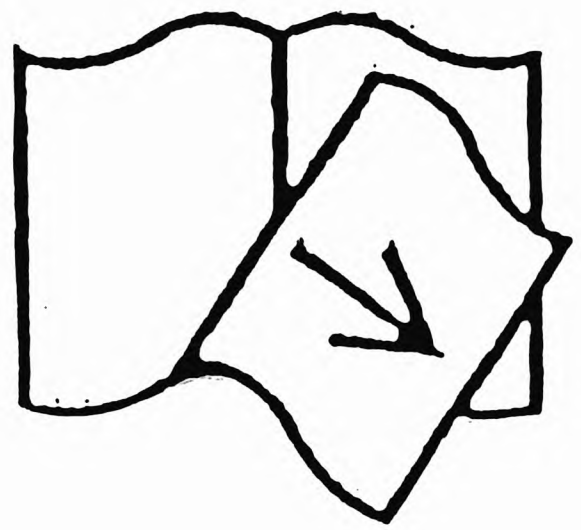
IV. Activation of egg metabolism to start development.

1.1.1. Mammalian Fertilisation.

The 17th century microscopist Leeuwenhoek first discovered sperm in 1678 but believed them to be parasitic animals living within the semen, hence the term spermatozoa, meaning "sperm animals". However, it was not until the 18th century that their role in fertilisation was discovered by Hertwig (1875), who demonstrated fertilisation when he observed the penetration of an egg by sperm and subsequently the union of male and female nuclei. The development and introduction of the electron microscope, in the middle of this century allowed scientists to gain a better insight and understanding of the sperm and its ultrastructural features.

Sperm released from the male reproductive tract are not immediately able to fertilise an egg (oocyte), but require a period of time to mature within the female reproductive tract (Austin, 1951; Chang, 1951). The series of alterations that occur during this period of maturation is termed capacitation and is essential for all mammalian sperm studied. The process of capacitation, the length of time taken is species-specific (Bedford, 1970), prepares the sperm to undergo the acrosome

PAGES MISSING
NOT AVAILABLE



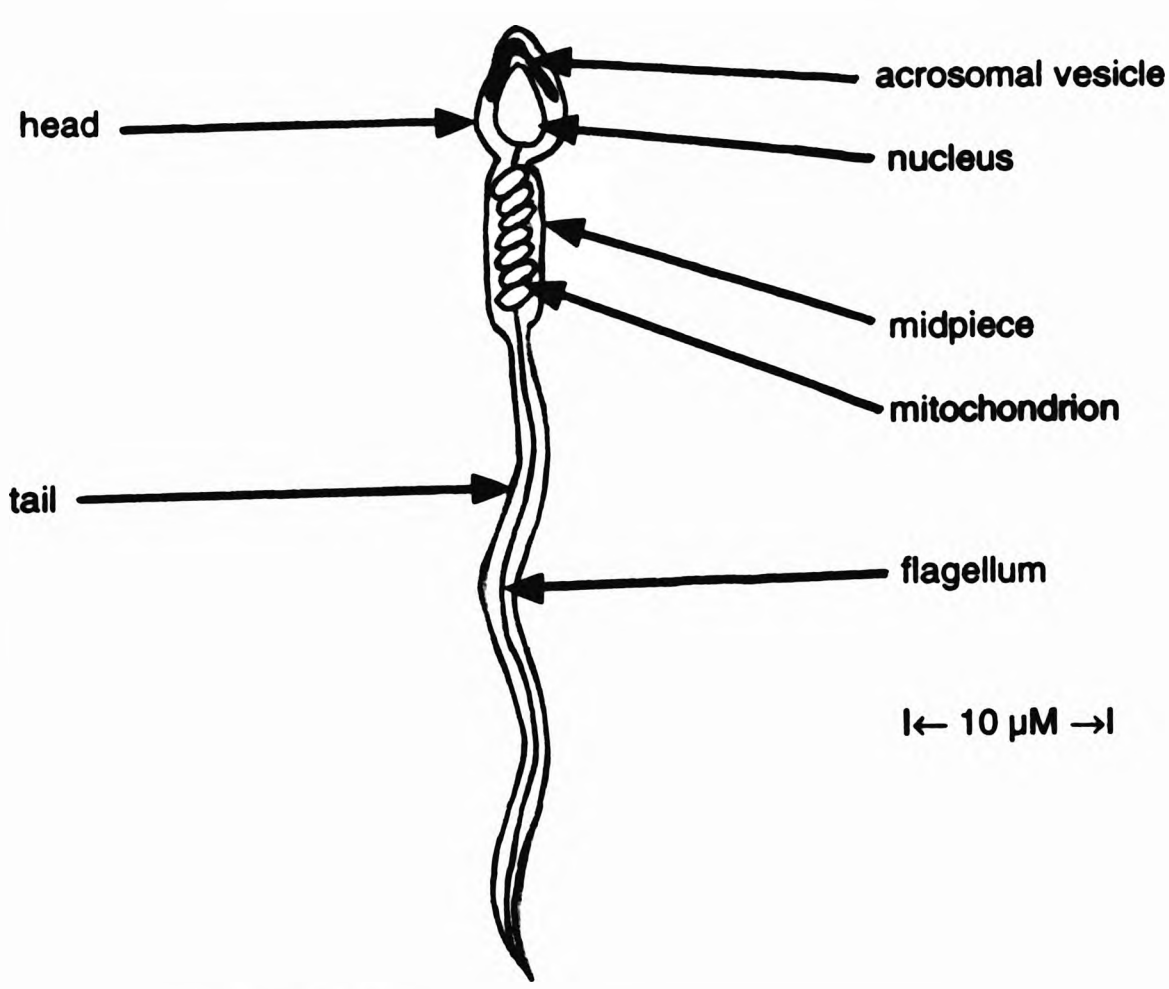
into the lumen of the seminiferous tubules. They subsequently pass into the epididymis, a coiled tube overlying the testes, where they are stored.

Typical mammalian spermatozoa (Figure 1.I) are "stripped down cells" equipped with strong flagella to propel them through aqueous media. They do not contain cytoplasmic organelles such as ribosomes, endoplasmic reticulum, or Golgi apparatus which are unnecessary for the task of delivering the DNA to the egg. Sperm do contain numerous mitochondria located in the midpiece and these organelles provide energy to power the flagella. Sperm usually consist of two morphologically and functionally distinct regions enclosed by a single plasma membrane:

I. The head, containing an unusually highly condensed haploid nucleus and a specialised secretory vesicle called the acrosome vesicle which contains hydrolytic enzymes that help the sperm penetrate the outer coat of the egg.

II. The tail, which propels the sperm to the egg and helps it burrow through the egg coat.

Figure 1.1. Structure of a typical mammalian spermatozoid.



1.1.1.2. Epididymal Maturation.

Mammalian sperm leaving the testis do not have the ability to fertilise eggs but gain this ability during the slow passage through the epididymis in a process called epididymal maturation (Bedford, *et al.*, 1973). The site where sperm begin to acquire the ability to fertilise eggs varies from species to species; for example, boar spermatozoa gain this ability in the distal segment of the caput epididymis, whereas, rat spermatozoa gain their fertilising ability in the corpus epididymis. It is unlikely that all the spermatozoa in a population gain the ability to fertilise an egg at the same time, some will acquire this ability faster than others (Yanagimachi, 1988). In general, it is not until spermatozoa enter the caudal epididymis that the majority of them gain their full fertilising potential.

The most striking change that occurs in spermatozoa during epididymal

maturation is the onset of the ability to move. Spermatozoa isolated from the caput region of the epididymis are either immotile or display a slight twitching motion and have a reduced metabolic rate, whereas sperm isolated from the caudal epididymis display progressive forward motility (Bedford, 1974; Hoskins, *et al.*, 1978; Brandt *et al.*, 1978). Progressive forward motility is an essential requirement for fertilisation. The initiation of motility in sperm as they pass through the epididymis involves physiological, biochemical and morphological changes (Bedford, 1974). The composition of the fluid in the lumen of the epididymal tubule differs from one region to another and these differences have been implicated in sperm maturation. For example, the concentrations of carnitine, glycerophosphorylcholine, inositol, Na^+, K^+ and Ca^{2+} vary in different parts of epididymis (Majumder, *et al.*, 1990). Sperm maturation also involves changes in membrane proteins (Olson and Danzo, 1981) and phospholipids (Voglmayr, 1975).

1.1.2. ***Fucus serratus.***

Fucus is a genus of a very common brown algae found mainly in the intertidal regions of rocky shores, all round the British coast. The plants are attached to rocks by means of a specialised basal disc called the holdfast and they vary considerably in size according to the conditions under which they grow. Under exposed and rather dry conditions near high tide marks they rarely extend more than six inches in length but under conditions where they are more completely immersed, and according to their age they may be anything up to three feet in length. The thallus of *Fucus serratus* is dark brown in colour and dichotomously branched, the lower part being narrow and almost round while the upper parts are flatter and broader with a thick midrib. As the

plants become mature the tips of the branches generally swell to form receptacles and in these many flask-shaped cavities (conceptacles) develop (Figure 1.II). The mucilage filled conceptacles contain the reproductive cells and open to the exterior via an ostiole. Male gametes (sperm) are formed in small saclike antheridia which develop as hairs lining the conceptacle; female gametes (eggs) are produced in larger sacs called oogonia, which arise from the conceptacle wall. Although *F. serratus* and *F. ceranoides* are dioecious, that is conceptacles on a single plant contain either antheridia or oogonia, some species of *Fucus,* for example, *F. spiralis, F. distichus* and *F. viroides* are monoecious where conceptacles on a single plant contain both antheridia and oogonia (Powell, 1963).

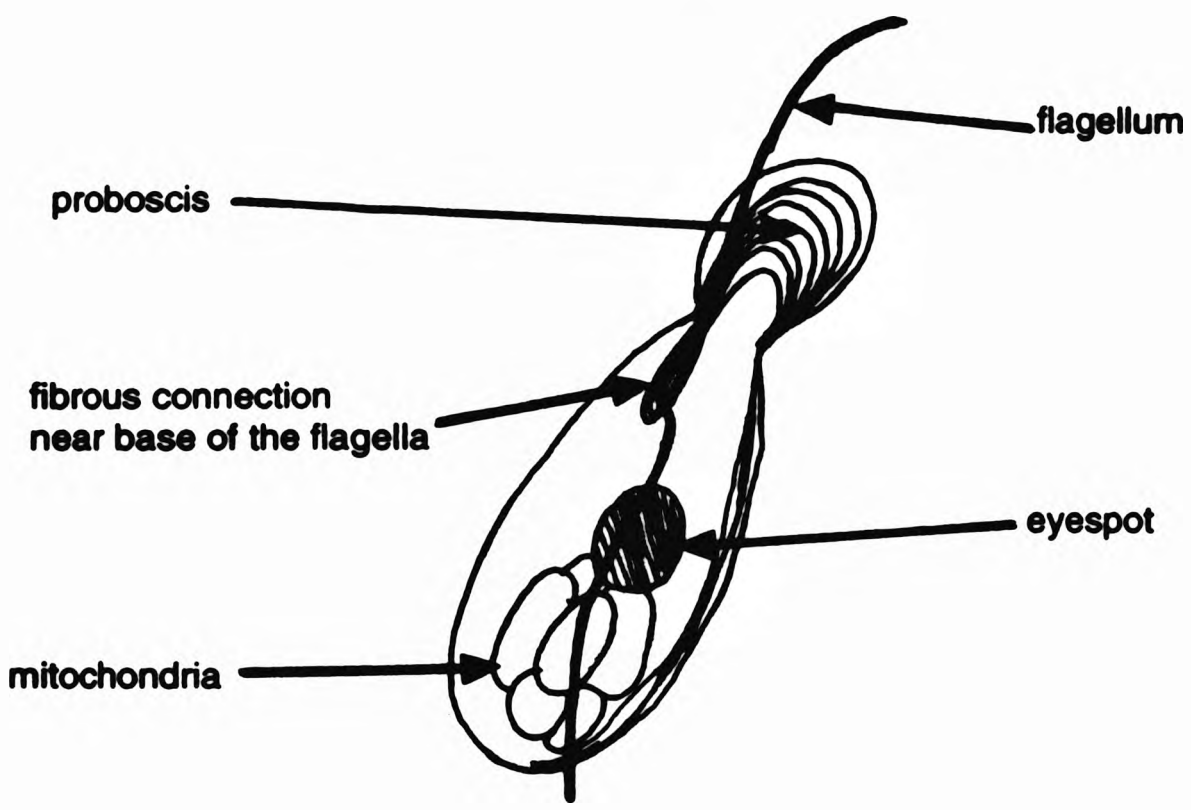
All *Fucus* plants are diploid sporophytes and the first nuclear division in oogonia and antheridia is meiotic. In oogonia a single mitosis follows this meiotic division resulting in eight (haploid) eggs, while in antheridia four mitotic divisions result in sixty-four spermatozoids (McCully, 1968). Individual oogonia and antheridia have three cell walls which are broken down to release eggs and sperm respectively. The outermost of three oogonial wall layers breaks at maturity and the eight eggs enclosed in the two inner layers are released into the sea through the conceptacle ostiole. The two outer wall layers of the antheridia also become disorganised at maturity releasing the sixty-four sperm into the sea still enclosed in an inner wall. Oogonia and antheridia are released at low tide and as the incoming tide rises the remaining wall layers dissolve releasing the eggs and sperm.

Figure 1.II. Photographs of *Fucus serratus* plants.

1.1.2.1. Gametes of *Fucus serratus.*

The liberated spherical eggs are about 80 µM in diameter, are non-motile and are enclosed in a plasma membrane (Callow, *et al.*, 1985). Newly released spermatozoids are pear shaped and are approximately 5 µM long (Friedmann, 1961). Sperm contain a large nucleus, a single chloroplast, several mitochondria and two flagella (Manton and Clarke, 1956) (Figure 1.III).

Figure 1.III. Diagrammatic representation of a spermatozoid of *Fucus serratus.*



The longer posterior flagellum is about seven times the length of the sperm body. The anterior flagellum bears helical rows of fine hairlike structures called mastigonemes (Manton and Clarke, 1956) and it remains erect. Spermatozoa are bright orange in colour due to carotenoid accumulation in a specialised region of the chloroplast known as the eyespot which enables sperm to swim towards diffuse illumination and away from intense light. The anterior end of the sperm body is extended into a proboscis, a flattened structure composed of 13 microtubules

connected to the flagella apparatus. These microtubules arise in the region of the anterior flagellar root and extend around one side of the cell just beneath the plasma membrane passing behind the eyespot and ending near the base of the cell (Manton and Clarke, 1956; Berkaloff and Rouseau, 1979; Callow, *et al.*, 1985). The function of the proboscis remains unknown, although it has been suggested that it may play a role in the attachment of the sperm to the egg. Manton (1969) and Friedmann (1961) noted that it was always pointed towards the egg surface. Unlike mammalian sperm those from *Fucus* have no acrosomal vesicle.

1.1.2.2. ***Fucus serratus* Fertilisation.**

It was demonstrated by Thuret as early as 1854 that *Fucus* sperm cells are attracted and excited by the eggs and form a swarming 'halo' around them. The initial fertilisation step for *Fucus* is mediated by the eggs secreting a pheromone-like sperm chemo-attractant and the development of a strong chemotactic response by the sperm. This chemo-attractant is volatile and non species specific; for example chemo-attractant secreted by eggs from both *F. serratus* and *F. vesiculosus* are equally effective in attracting sperm not only from each other but also sperm from *F. spiralis*. Cooke and colleagues (1951) found a variety of hydrocarbons such as n-hexane, ethers and esters were capable of mimicking the natural chemo-attractant. Further work by Hlubeck and co-workers (1970) showed that traces of n-hexane existed in the fruiting tips of female *F. vesiculosus* and these workers suggested that this substance was the native chemo-attractant. Between 1973 and 1979 Müller and co-workers isolated and characterised a conjugated hydrocarbon, 1,3 trans, 5-cis-octatriene; C_8H_{12} (fucoserraten), from *F. serratus* and found that this compound was a highly potent sperm attractant of sperm from both *F. serratus* and *F. vesiculosus* at concentrations of 10^{-6} M. These workers (1979) later showed that fucoserraten was

also secreted by the eggs of *F. vesiculosus* and that sensitivity of sperm to this compound was three orders of magnitude greater than it was to n-hexane.

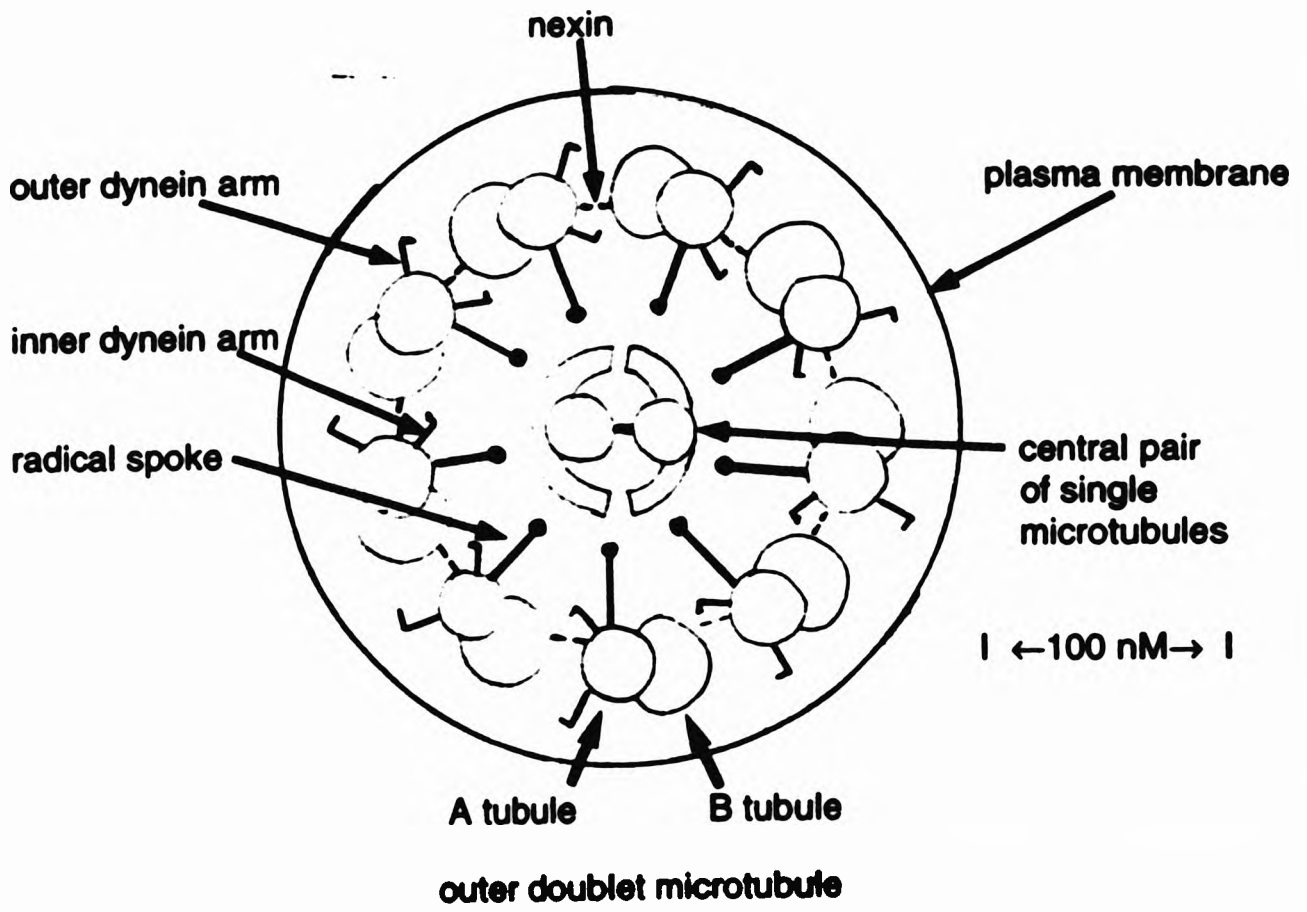
Sperm shape changes during the chemotactically mediated approach to the egg surface. The body becomes more elongated and "comma-like" and the concave side of this arched body and hence the proboscis faces the egg surface. Sperm make contact with the egg membrane by "probing" the surface with the tips of their anterior flagella (Friedmann, 1961) and following specific binding, fusion of the flagella and egg membranes occurs. Only a few sperm of *Fucus* bind to the surface of the egg indicating either, that the sperm receptors on the egg surface are not homogeneously distributed or, that the attachment of the first sperm somehow prevents further sperm from binding (polyspermy). This latter effect could be induced by changes in receptor distribution, surface potential or some other mechanism. After gamete fusion, the resulting zygote releases the ß-linked polypuronide alginic acid from a large number of sub-plasmalemmal vesicles. The secretion of this compound commences at the point of sperm entry and continues until the whole zygote is encased in a wall of alginic acid (Evans *et al.*, 1982). After twenty-four hours the oospore nucleus divides and a cell wall is laid down between the daughter nuclei. The lower of the two cells develops into the holdfast while the rest of the plant grows from the upper cell.

1.2. Motility of Spermatozoa

There is very little information concerning the mechanisms that initiate motility and respiration in the spermatozoa of *Fucus serratus* therefore thoughout this thesis reference will primarily be made to literature on mechanisms operating in mammalian and sea urchin spermatozoa.

The motility of spermatozoa arises from their motile tails which are long flagella whose central axoneme emanates from a basal body situated just posterior to the nucleus (Lindemann and Rikmenspoel, 1972). The bending of the axoneme, which consists of two central singlet microtubules surrounded by nine evenly spaced microtubule doublets, produces the movement of flagella. The flagella of some sperm, including those of mammals, differs from this model in that the usual 9 + 2 pattern of the axoneme is further surrounded by nine outer dense fibres of unknown composition (Figure 1.IV).

Figure 1.IV. Schematic representation of a flagellum.



These dense fibres are stiff and noncontractile, and it is not known what part they play in the active bending of flagella. The microtubules of the axoneme are linked to structures that generate force and enable it to produce wavelike movements. The most important of these structures are the dynein arms which project from the microtubule doublets and interact with adjacent doublets to produce bending. Links composed of the protein nexin hold adjacent microtubule doublets together and prevent sliding between adjacent doublets. Dynein is a large protein complex containing two or three globular heads depending upon the species and each head

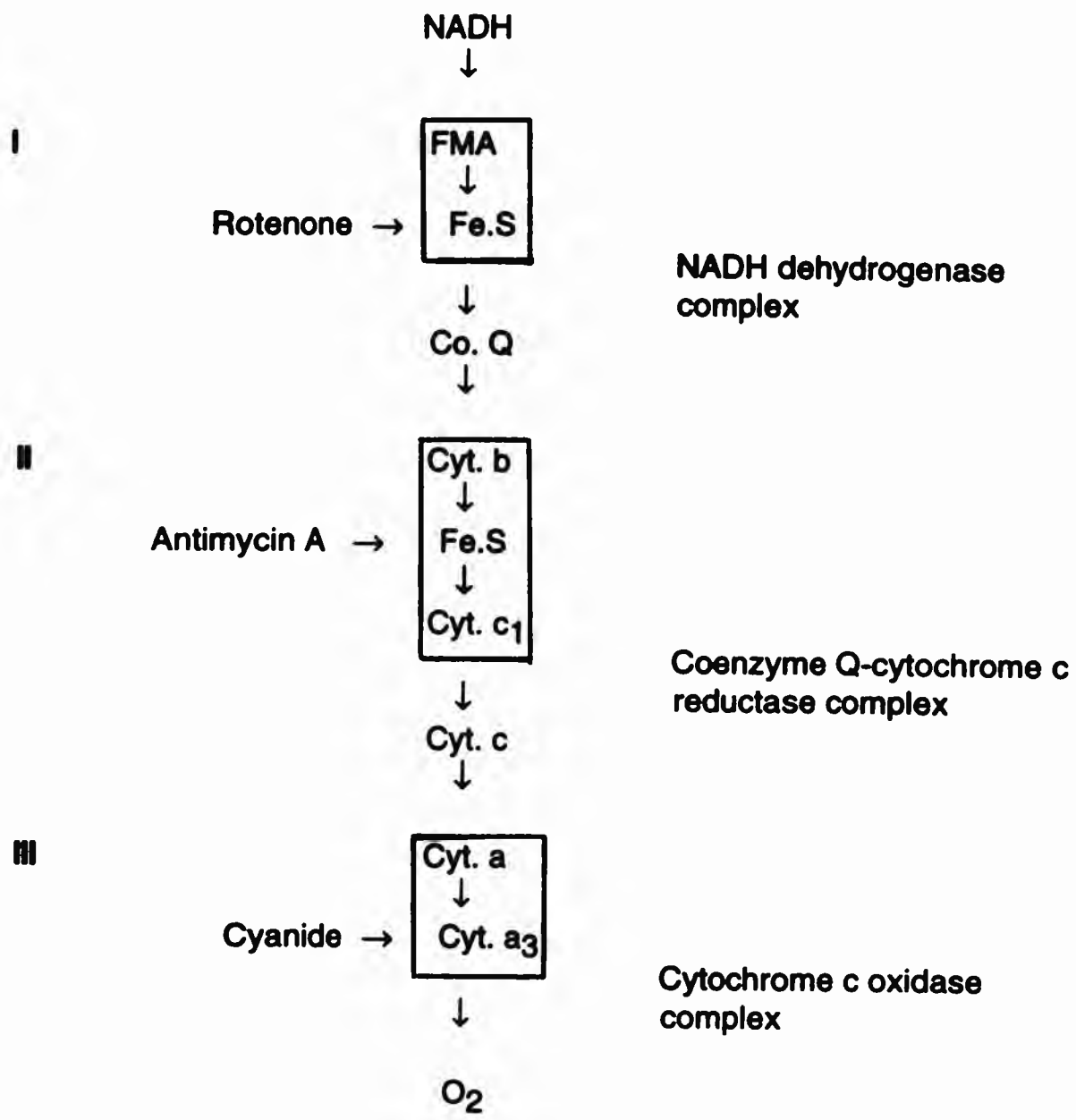
has ATPase activity, that is they catalyse the hydrolysis of ATP. The active bending of each flagellum is caused by the sliding of the adjacent microtubule doublets past one another and this movement is powered by the hydrolysis of ATP generated by highly specialised mitochondria in the anterior part of the sperm tail called the midpiece.

1.3. Energy Requirements for Motility and Respiration.

The control of respiration in spermatozoa has been most widely studied in sea urchin since these sperm are easily obtainable in large quantities and, like other spermatozoa, are devoid of complex machinery to replicate DNA or synthesise proteins. Additionally sea urchin sperm appear to have no glycogen and ATP is primarily formed by the respiration of a single mitochondrion (Christen, *et al.*, 1982; 1983). In mammalian spermatozoa numerous mitochondria are wrapped tightly around the flagella thus providing ATP directly to these high ATP consuming sites.

Oxidative phosphorylation is the process in which ATP is formed as electrons are transferred from NADH or $FADH_2$, high energy molecules formed in the tricarboxcyclic acid cycle, to O_2 by a series of electron carriers (Figure 1.V).

Figure 1.V. Sequence of the electron transport chain.



There are three major membrane-bound enzyme complexes in the pathway of electrons transferred from NADH to O_2 (indicated in boxes in Fig. 1.V).

I The NADH dehydrogenase complex: This complex accepts electrons from NADH and passes them through a flavin and at least five iron-sulphur centres to coenzyme Q (ubiquinone), a lipid-soluble molecule, that transfers electrons to the second complex.

II The coenzyme Q-cytochrome c reductase complex: This complex contains cytochromes b and c in addition to an Fe.S protein and accepts electrons from ubiquinone and passes them on to cytochrome c, a small peripheral protein that

carries electrons to the third complex.

III The cytochrome c oxidase complex: This complex contains cytochromes a and a_3 and accepts electrons from cytochrome c and passes them to O_2.

The three major complexes in the electron chain are also the sites where the transport of electrons is coupled with the generation of ATP a process referred to as oxidative phosphorylation. Information on the carrier sequence has been obtained by the use of inhibitors that block specific transfer steps in the chain and the site of action of some of these inhibitors is shown in Fig. 1.V. Inhibitors used to determine whether the respiration of a cell occurs through the whole span of the electron transport chain include:

a) rotenone, a plant toxin which specifically inhibits electron transfer within the NADH dehydrogenase complex and prevents the generation of a proton gradient at site I.

b) antimycin A, an antibiotic isolated from *Streptomyces griseus* which inhibits electron flow between cytochromes b and c_1 and prevents ATP synthesis coupled to the generation of a proton gradient at site II.

c) cyanide which blocks the electron flow between the cytochrome c oxidase complex and O_2. Thus phosphorylation coupled to the generation of a proton gradient at site III does not occur.

Oxidative phosphorylation is coupled tightly with the requirement for cellular ATP such that electron flow from organic fuel molecules to O_2 is adjusted to the energy needs of the cell by ADP-mediated respiratory control. Studies of oxidative phosphorylation have been greatly aided by the availability of various agents that affect the process in different ways. Two different types of compounds are known that are either classified as uncouplers or as inhibitors of oxidative phosphorylation.

Uncouplers of oxidative phosphorylation, for example dinitrophenol (DNP) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), function by dissociating the electron transport process from the generation of ATP with which it is normally

tightly coupled. As stated previously the cellular requirement for ATP controls the rate of oxidative phosphorylation. An uncoupling agent frees electron transport from this control and allows it to proceed at an uncontrolled pace unaccompanied by ATP production. The result is an excessive consumption of O_2 and an unimpeded utilisation of substrate and dissipation of energy as heat.

Inhibitors of oxidative phosphorylation, for example oligomycin, are agents which prevent both O_2 consumption and ATP generation, but they do so without directly inhibiting the electron carriers of the transport chain and can thereby be differentiated from inhibitors of electron transport such as rotenone, antimycin A or cyanide. These inhibitors interfere directly with the process of ATP synthesis.

Sperm motility is dependent on an adequate supply of ATP and this is produced either by glycolysis or by oxidative phosphorylation, depending on the species and the availability of substrates (Lardy and Phillips, 1941). The mitochondria of mammalian sperm are located in the midpiece of the tail and, therefore, for acquisition of motility throughout the entire length of the flagellum, ATP or a high energy equivalent must diffuse or be actively transported down the axoneme to the dynein ATPases (Gibbons, 1982). Originally it was thought that mitochondrial ATP was sufficient to produce full motility in both sea urchin and bovine spermatozoa (Adam and Wei, 1975). However, observations of the motility of sea urchin sperm indicate that ATP diffusion from the single mitochondrion, would not support maximal motility particularly in the distal portion of the flagellum (Tombes and Shapiro, 1985). In the spermatozoa of sea urchin, the high energy compound, creatine phosphate is involved in the establishment of the pool of ATP which is used for motility (Tombes and Shapiro, 1985; Quest and Shapiro, 1991). The phosphocreatine is formed at the junction of the mitochondria and tail by creatine kinase from creatine and mitochondrial ATP.

$$\text{Creatine} + \text{ATP} \xleftrightarrow{\text{creatine kinase}} \text{Phosphocreatine} + \text{ADP}$$

This phosphocreatine diffuses down the tail where it reacts with ADP in the presence of a different creatine kinase isoenzyme to form ATP and creatine. The latter reaction is assumed to occur in close proximity to dynein ATPases (Schoff, *et al.*, 1989). There is no evidence of a similar system existing in bovine sperm, which have virtually no creatine kinase activity (Tombes and Shapiro, 1989) and in these sperm, which contain numerous mitochondria, ATP is believed to diffuse down the flagellum in sufficient quantities to provide energy for motility as well as for ion pumps and other ATP-utilising systems. In general, mammalian sperm do not have an active system for transporting ATP down the flagellum in response to increasing demands and hence changes in motility are limited by the efficiency of this diffusion of ATP down the tail (Schoff, *et al.*, 1989). Another enzyme involved in ATP metabolism, which appears to be associated with sperm motility is adenylate kinase. This enzyme activity has been detected in a variety of cilia and flagella, including flagella from algae (Brokaw, 1961), sea urchin sperm (Tombes and Shapiro, 1985) and bovine spermatozoa (Lindemann and Rikmenspoel, 1972). However the activity of adenylate kinase in sea urchin sperm is insufficient to allow maximal motility in the absence of the phosphocreatine shuttle (Tombes, *et al.*, 1987). Adenylate kinase is an ubiquitous enzyme that can produce either ADP or stoichimetric amounts of ATP and AMP depending on the intracellular concentrations of the three nucleotides.

$$2ADP \xleftarrow{\text{adenylate kinase}} ATP + AMP$$

In mammalian sperm adenylate kinase forms ATP in response to high ADP levels that develop when motility is stimulated after capacitation, the maturation process that occurs as sperm travel through the female tract, or prior to fertilisation (Yanagimachi, 1981). The AMP produced by this reaction diffuses back to the

mitochondria in the midpiece where it is rephosphorylated by a mitochondrial adenylate kinase, located between the inner and outer mitochondrial membranes, to form ADP. The location and specific function of adenylate kinase in sperm tails is not fully understood. One proposal is that the enzyme is located in the vicinity of the dynein ATPases, thus ensuring the availability of additional energy at times of increased demand (Schoff, *et al.*, 1989).

In summary sea urchin sperm have both creatine kinase and adenylate kinase activity. On the other hand bovine sperm and sperm from other species, including rabbit and human, as well as the flagellated alga *Chlamydomonas reinharditii*, have virtually no creatine kinase activity (Schoff, *et al.*, 1989). In these sperm which lack creatine kinase, adenylate kinase activity may replace the phosphocreatine shuttle, by increasing the supply of utilisable phosphoryl groups to dynein ATPase.

Spermatozoa are propelled forward by the beating of their flagella. ATP provides the energy for this movement and energy coupling is effected by dynein ATPase which is localised within the axoneme of the flagella (Gibbons, *et al.*, 1976). Thus, the motility and respiration of spermatozoa have the potential for being linked (Christen, *et al.*, 1982). ATP generated by respiration is used for motility and the ADP produced as a result of motility is required for respiration of tightly coupled mitochondria (Brokaw and Benedict, 1968; Christen, *et al.*, 1982). Therefore agents that affect respiration may also affect motility.

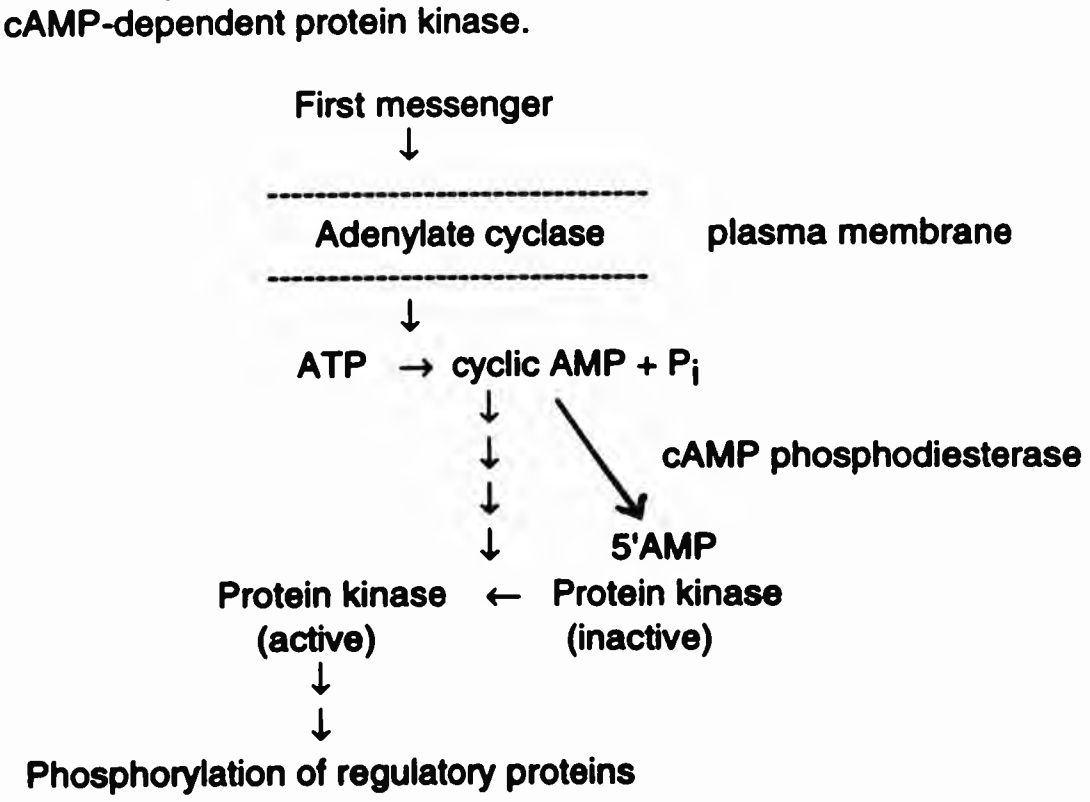
1.4. Regulation of Sperm Motility.

It is known that mammalian spermatozoa acquire the capacity to undergo forward progressive motility as they pass along the epididymis and that sea urchin spermatozoa become motile upon release into sea water. What remains unclear is regulatory mechanisms that enable sperm to acquire this motility. There are four mechanisms that may be involved in the initiation and regulation of sperm motility: the production of cyclic nucleotides such as cAMP, the activation of protein kinase C though the inositol phosphate pathway, changes in calcium concentration and alterations of intracellular pH.

1.4.1. Adenosine 3',5'- cyclic Monophosphate.

Adenosine 3',5'- cyclic monophosphate (cAMP) has been shown to have a regulatory effect in a wide variety of biological systems (Sutherland, *et al.*, 1968).

Figure 1.VI. Schematic representation of the regulation of physiological events through cAMP and cAMP-dependent protein kinase.



In most cells hormones activate a membrane-bound enzyme, adenylate cyclase which leads to increased synthesis of cAMP. A direct correlation between the activation of adenylate cyclase and stimulation of motility has been shown in porcine spermatozoa (Okamura, *et al.*, 1985).

Bicarbonate, over the physiological concentration range (1 → 50 mM) has been shown to activate both adenylate cyclase and motility in mammalian spermatozoa from several species including bovine, mouse, porcine and human as well as in sea urchin spermatozoa (Okamura, *et al.*, 1985), thus indicating that bicarbonate-mediated activation of adenylate cyclase and the consequent rise in intracellular cAMP levels plays a critical role in the regulation of sperm motility (Okamura, *et al.*, 1985).

The cellular levels of cAMP not only depend upon the rate of cAMP synthesis but also on the rate of its degradation catalysed by cAMP-specific phosphodiesterase, which converts cAMP → 5'AMP. The presence of cAMP in spermatozoa was demonstrated in sea urchin sperm by Gray and co-workers (1971), and Casillas & Hoskins (1970) observed thyroxin mediated activation of adenyl cyclase in the presence of caffeine, a cAMP phosphodiesterase inhibitor, in the spermatozoa of monkeys. The latter workers also showed that inhibitors of cAMP-specific phosphodiesterase, for example caffeine and theophylline markedly increased not only the intracellular levels of cAMP but also the respiration and motility of bovine epididymal spermatozoa.

A more direct method of assessing whether cAMP has an effect on the metabolism and motility of intact spermatozoa is to incubate them in its presence. However, cAMP has a low degree of penetrability and is rapidly hydrolysed by phosphodiesterase (Garbers, *et al.*, 1971). The N^6,2'-O-dibutyryl derivative of cAMP (dbcAMP) was discovered to be a more effective way in penetrating cell membranes, presumably because it is more lipid soluble and more resistant to hydrolysis (Robison, *et al.*, 1968). The addition of dbcAMP to bovine spermatozoa caused a large

stimulation in their respiration and motility (Garbers, *et al.*, 1971).

Thus indirect evidence using inhibitors of cAMP-specific phosphodiesterase and more direct evidence using analogues of cAMP has shown that cAMP stimulates respiration and motility of spermatozoa. Further the intrasperm levels of cAMP have been shown to increase during epididymal maturation of bovine spermatozoa (Hoskins, 1973). These increased levels of cAMP play a critical role in both the initiation and maintenance of motility of epididymal sperm (Majumder, *et al.*, 1990).

The cAMP-dependent regulation of sperm metabolism and motility is believed to be mediated through the control of a cAMP-dependent protein kinase which when activated leads to the phosphorylation of regulatory proteins, see Figure 1.VI (Tash and Means, 1982a & b). Results from various studies have indicated that cAMP-dependent protein kinase is present in the cytosol of mammalian spermatozoa including bovine (Hoskins, *et al.*, 1972) and rat (Horowitz, *et al.*, 1984; 1989), as well as in the spermatozoa of sea urchin (Lee and Iverson, 1976). It has been documented that the activity of cAMP-dependent protein kinase increases as mammalian sperm travel through the epididymis further suggesting that cAMP is involved in the initiation and maintenance of motility (Hoskins, *et al.*, 1974).

The phosphorylation state of several proteins, which were first identified in dog sperm using [^{32}P] ATP, is closely linked to cAMP-dependent stimulation of flagella motility (Tash and Means, 1982a & b). Tubulin and the dynein heavy chain of the axoneme are two proteins reported to be phosphorylated in a cAMP-dependent manner (Tash and Means, 1982a & b; 1983; Mohri, 1993). Detergent permeabilized models of starfish and sea urchin sperm which had been centrifuged to remove material released by the detergent treatment had lost their ability to be stimulated by cAMP (Ishiguro, *et al.*, 1982). This suggested that a factor which stimulated flagella motility had been released from the sperm by detergent treatment and was present in the supernatant. The activity of this factor as its action was also blocked by protein

kinase inhibitors suggesting that the activity of cAMP-dependent protein kinase was necessary for its activation. A protein was subsequently separated from cAMP-dependent protein kinase, using supernatant from permeabilized cells, on a DEAE chromatography column. A similar detergent-extractable cAMP-dependent, motility-stimulating protein factor was also found in dog, human, rat, pig and bull spermatozoa (Tash and Means, 1988) and was also located from other axoneme enhanced tissue such as trachea and retina. When a detergent extract of dog spermatozoa was incubated with [^{32}P] ATP in the presence of cAMP-dependent protein kinase five major phosphoproteins were identified and four of these proteins demonstrated marked cAMP-dependence for their phosphorylation. The phosphoprotein which showed the greatest cAMP-dependent incorporation of ^{32}P had a molecular weight of 56,000 daltons (Tash, *et al.*, 1984). This cAMP-dependent phosphoprotein was also found in sperm of other species and further, the phosphoprotein from one species could activate extracted flagella from other species (Tash and Means, 1988). Tash and co-workers (1984) named this protein factor axokinin and showed that it is an ubiquitous axoneme-specific protein. The use of a selective inhibitor of cAMP-dependent protein kinase, N-[2 (methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) confirmed that the phosphorylation of axokinin is required for the initiation of motility in the flagella and that this phosphorylation is catalysed by cAMP-dependent protein kinase (Tash *et al.*, 1986).

1.4.2. Inositol Phosphate Pathway.

The receptor-mediated hydrolysis of membrane-bound inositol phospholipids is known to be a common mechanism for the transduction of extracellular signals across the plasma membrane of somatic cells (Berridge, 1987a & b). However the precise physiological and biochemical role of phospholipases in mammalian spermatozoa is unclear.

The hydrolysis of phosphatidyl inositol by phosphoinositol-specific phospholipase C (PI-PLC) has been reported in human sperm (Atreja and Anand, 1985), goat sperm (Bansal and Atreja, 1991) as well as in sea urchin sperm (Takei, *et al.*, 1984). The activity of PI-PLC changes during the epididymal maturation of goat spermatozoa with a 6.5 fold increase in the activity of this enzyme in the sperm and a 4 fold decrease in its activity in the fluid of the lumen of the epididymis during passage from the caput to the caudal epididymis (Bansal and Atreja, 1991). Thus these workers concluded that there is an uptake of PI-PLC by the spermatozoa from the fluid of the lumen of the epididymis.

PI-PLC hydrolyses phosphoinositides to generate two products, viz: inositol trisphosphate and diacylglycerol which can be converted to glycerol which in turn can result in the production of triglycerides when combined with fatty acids. These may be used as an energy source by spermatozoa during epididymal maturation (Atreja and Anand, 1985). More importantly inositol trisphosphate and diacylglycerol may also act as second messengers in various tissues and cells (Berridge, 1984). Inositol trisphosphate, a small water-soluble molecule mobilises intracellular Ca^{2+} from the endoplasmic reticulum thus increasing the concentration of Ca^{2+} in the cytosol. Ca^{2+} has been shown to play a role in mammalian sperm capacitation and activation of motility at the time of fertilisation (Breitbart, *et al.*, 1985). Also Ca^{2+} is required for the activation of almost all phospholipases (Bansal and Atreja, 1991). Diacylglycerol can

undergo further biochemical changes to form arachidonic acid, which in turn can be used in the synthesis of prostaglandins and related signalling molecules, or it can activate a specific protein kinase, namely protein kinase C (PKC) which in turn catalyses the phosphorylation of a number of proteins with different functions in the cell (Nishizuka, 1986). Diacylglycerol together with the phospholipid phosphatidylserine binds to PKC, thereby increasing the affinity of the enzyme for Ca^{2+} such that the PKC is activated at low concentrations of Ca^{2+} normally found in the cytosol. In many cells activation of PKC is most probably achieved by the co-operative effect of diacylglycerol and an increase in intracellular Ca^{2+} brought about by the action of inositol trisphosphate. One of the actions of PKC in many animal cells is the catalysis of phosphorylation and thereby activation of the plasma membrane Na^{+}/H^{+} exchanger that controls intracellular pH (Stiffert and Akkerman, 1988). An increase in this pH has been implicated in the initiation of sperm motility (Christen, *et al* 182; 1983, Lee, 1984a & b).

If the phosphoinositol phosphate pathway is involved in sperm motility then inhibitors of PKC should inhibit motility and stimulators of the kinase should induce motility. Rotem and colleagues (1990a & b) have shown that human sperm motility was stimulated by the phorbol diester 12-O-tetradecanayl phorbol-13-acetate (TPA) and β-phorbol diester which are activators of PKC but not by the biologically inactive α -phorbol diester. On the other hand the PKC inhibitor straurosporine inhibited sperm motility. These results together with the observation that the permeable analogue of diacylglycerol, namely 1-oleoyl-2-acetyl glycerol (OAG), also enhanced the motility of human sperm led these workers to conclude that PKC was involved in flagella motility (Rotem, *et al.*, 1990a & b).

It is of interest that in hamster spermatozoa analogues of diacylglycerol, for example OAG and activators of PKC such as phorbol 12-myristate 13-acetate (PMA), also cause an increase in the levels of cAMP providing Ca^{2+} is present (Visconti and

Tezon, 1989). In the presence of bicarbonate, which would produce an increase in intracellular pH, PMA induced a 40 fold rise in cAMP levels over those produced in the presence of PMA alone (Visconti, *et al.*, 1990). The involvement of extracellular bicarbonate as well as PKC in the elevation of cAMP was conclusively demonstrated by Visconti and colleagues (1990) who incubated spermatozoa with either 1 mM diisothiocyanate stilbene 2,2'-disulfonic acid (DIDS) or 1 mM acetamido 4'-isothiocyanate stilbene 2,2'-disulfonic acid (SITS) which are well known inhibitors of the bicarbonate/chloride exchanger in several systems (Hoffman, 1986). These agents caused an 87 → 95% inhibition of PMA-induced cAMP accumulation. These results suggest the presence of an anion transport mechanism in spermatozoa and that the transport of bicarbonate across the plasma membrane as well as the activity of PKC could be linked to the regulation of cAMP synthesis (Visconti, *et al.*, 1990). Thus it would appear that second messenger systems are interdependent in activation of sperm motility.

1.4.3. <u>Calcium.</u>

The role of extracellular Ca^{2+} in regulating the motility of mammalian and invertebrate spermatozoa is unclear. Studies on membrane-permeabilized spermatozoa have shown that low concentrations of Ca^{2+} (10^{-9} M) produce symmetrical waveforms of the flagella whereas higher concentrations (10^{-6} M) produce either assymetrical waveforms or inhibit flagella motility totally (Gibbons and Gibbons, 1973). The presence of external Ca^{2+} stimulates the motility of hamster spermatozoa (Morton, *et al.*, 1974), inhibits the motility of dog spermatozoa (Tash and Means, 1982a & b) and has no effect on spermatozoa of guinea pig, mouse or rabbit (Vijayaraghavan and Hoskins, 1990). The reason for these species differences remain

unclear as little is known about the mechanisms regulating the entry of Ca^{2+} into the cell and the subsequent effects on motility.

The intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$) has been shown to have a role in the regulation of spermatozoa motility (Garbers and Kopf, 1980). In mammalian spermatozoa, systems that regulate $[Ca^{2+}]i$ involve the Na^+/Ca^{2+} exchanger (Rufo, *et al.*, 1984), the ATP-dependent Ca^{2+} pump (Bradley and Forester, 1980) and the voltage-dependent Ca^{2+} channel (Breitbart, *et al.*, 1985; 1990) of both the mitochondrial and the plasma membranes.

In sea urchin spermatozoa Ca^{2+} is required for the acrosome reaction (Dan, 1954) and the motility of these spermatozoa decreases in the presence of the cation chelator EGTA suggesting that Ca^{2+} plays a role in their activation (Young and Nelson, 1974). Both motility and respiration increase on the addition of the Ca^{2+} ionophore A23187 to sperm incubated in Na^+-free artificial sea water (ONaASW), but the addition of this ionophore had no additional effect on either the percent of motile sperm or their rate of O_2 uptake when these sperm were suspended in artificial sea water (ASW) (Mita,1984). The rate of respiration and the percent of motile sperm were low when spermatozoa were incubated in ONaASW containing 10 mM $CaCl_2$, suggesting that the activation of sea urchin spermatozoa seen upon dilution into sea water is independent of external Ca^{2+} (Mita, 1984). Schackman and colleagues (1978) also reported that there was no uptake of Ca^{2+} following dilution of sea urchin sperm into sea water. The activation of sea urchin sperm is associated with an increase in intracellular pH, which is brought about by the activation of Na^+/H^+ exchange (Lee, *et al.*, 1983) and it is proposed that the resulting alkalisation may somehow cause the activation of respiration (Mita, 1984).

The Na^+/Ca^{2+} exchanger has been implicated in both the entry and removal of cellular Ca^{2+} and is therefore thought to play a role in Ca^{2+} homeostasis (Langer, 1982). In bovine ejaculated spermatozoa this exchanger is associated with Ca^{2+}

uptake which is blocked by caltrin, a low molecular weight protein found in seminal plasma. Caltrin binds to ejaculated bovine spermatozoa and inhibits Na^{+}/Ca^{2+} exchange activity and, thus, this protein may play a role in regulating the uptake of Ca^{2+} under physiological conditions (Rufo, *et al.*, 1984). The inhibitory effects on Ca^{2+} uptake of purified caltrin on epididymal bovine spermatozoa is reversed in the presence of the amiloride analogue, 3',4'-dichlorobenzamil, which is an activator of Ca^{2+} uptake (Breitbart, *et al.*, 1990). One possible explanation for these effects is that this analogue inhibits the Na^{+}/Ca^{2+} exchanger (Kaczorowski, *et al.*, 1985; Breitbart, *et al.*, 1990). However, 3',4'-dichlorobenzamil enhanced Ca^{2+} uptake even when added to sperm incubated in ONaASW indicating that stimulation is not due to inhibition of the Na^{+}/Ca^{2+} exchanger (Breitbart, *et al.*, 1990). On the other hand, the voltage-dependent Ca^{2+} channel blockers, nifedipin and diltiazem do inhibit the stimulation of Ca^{2+} uptake induced in spermatozoa by the amiloride analogue 3',4'-dichlorobenzamil suggesting that this stimulation of Ca^{2+} uptake may, at least partially, involve the activation of a voltage-dependent Ca^{2+} channel (Breitbart, *et al.*, 1990).

As stated previously the intracellular concentration of Ca^{2+} has a role in the regulation of the motility of spermatozoa (Garbers and Kopf, 1980). Changes in $[Ca^{2+}]i$ are known to occur during the epididymal maturation of mammalian spermatozoa but it is unclear how these changes come about as sperm are insensitive to hormones and other physiological agents which affect Ca^{2+} fluxes in other cell types (Hoskins, *et al.*, 1975). The major problem in investigating the mechanisms by which $[Ca^{2+}]i$ is regulated in spermatozoa has been the lack of suitable techniques to measure the intrasperm levels of Ca^{2+}. However, the introduction of Ca^{2+} sensitive fluorophores such as Quin-2 and Fura-2 have made the measurement of intracellular Ca^{2+} feasible in several somatic cells (Tsein, *et al.*, 1982; Grynkiewicz, *et al.*, 1985). Vijayaraghavan and Hoskins (1989) measured the

$[Ca^{2+}]i$, using both Quin-2 and Fura-2, in bovine spermatozoa and showed that internal Ca^{2+} levels are significantly higher when external Ca^{2+} is present. These workers found that the $[Ca^{2+}]i$ of these spermatozoa decreases as the amount of Quin-2 loaded into the sperm increases even in the presence of external Ca^{2+}. This decrease did not appear to be interfering with Ca^{2+} uptake as exogenous radio-labelled Ca^{2+} ($^{45}Ca^{2+}$) was taken up at the same rate and to the same extent in the presence or absence of Quin-2. This uptake of external $^{45}Ca^{2+}$ was inhibited by ruthenium red and by the mitochondrial oxidative phosphorylation uncoupler CCCP. However, external Ca^{2+} was not taken up by spermatozoa loaded with Quin-2 which had first been incubated in the absence of external Ca^{2+} (Vijayaraghavan and Hoskins, 1989). Further, when the metabolic inhibitors CCCP, antimycin A or rotenone were present during dye loading, the concentration of intracellular Ca^{2+} was similar to that observed in Ca^{2+} depleted spermatozoa. These results suggest that external Ca^{2+} may enter the spermatozoa through the mitochondria (Vijayaraghavan and Hoskins, 1989). Other workers have also shown that bovine caudal epididymal spermatozoa accumulate relatively large amounts of Ca^{2+} into their mitochondria when incubated in Ca^{2+} containing media (Babcock, *et al.*, 1976).

The capacity for Ca^{2+} uptake is inhibited in ejaculated bovine sperm when they come into contact with the secretions from seminal vesicles. These secretions contain two proteins, caltrin and BSPA1/A2 which have been shown to bind to sperm and reduce their capacity for Ca^{2+} uptake (Rufo, *et al.*, 1984; Vijayaraghavan and Hoskins, 1990). From these results it has been postulated that a decrease in intracellular Ca^{2+} levels may be involved in the initiation of motility during epididymal maturation (Vijayaraghavan and Hoskins, 1990). The $[Ca^{2+}]i$ is 6 times greater in caput epididymal sperm than in caudal epididymal sperm as measured with Fura-2 (Vijayaraghavan and Hoskins, 1990) and this higher Ca^{2+} content of caput epididymal sperm appears to be due to a higher rate of Ca^{2+} uptake by the mitochondria of these

sperm than that of caudal epididymal sperm. When the rate of mitochondrial Ca^{2+} uptake was measured in digitonin permeabilized sperm it was found to be 2 to 3 times greater in caput epididymal sperm than in caudal epididymal sperm.

Vijayaraghavan and Hoskins (1989; 1990) have proposed that external Ca^{2+} has access to sperm only via the mitochondria and mitochondrial Ca^{2+} is redistributed into the cytoplasm of the sperm as a function of pHi. When intracellular pH (pHi) was increased by the addition of NH_4Cl to caput epididymal spermatozoa a significant decrease in the $[Ca^{2+}]i$ was observed whereas acidification with pyruvic acid results in an increase in the $[Ca^{2+}]i$ (Vijayaraghavan and Hoskins, 1990). The intracellular pH of caput sperm is acidic (pH 5.8) compared with that of caudal sperm. This difference in pH could therefore account for the higher levels of Ca^{2+} found in the cytoplasm of sperm isolated from the caput region of the epididymis. The consequences of higher $[Ca^{2+}]i$ in caput epididymal sperm relative to the levels found in caudal epididymal sperm are twofold. Firstly, high intrasperm Ca^{2+} levels have been shown to inhibit flagella motility both in sperm that have had their membranes removed (Tash and Means, 1982a & b), and in intact sperm. This has been demonstrated using the Ca^{2+} ionophore A23187, which induces high $[Ca^{2+}]i$. On treatment with this compound the flagella motility of bovine epididymal spermatozoa was inhibited (Vijayaraghavan and Hoskins, 1990). Secondly, high intracellular Ca^{2+} levels may regulate cAMP, this in turn results in the low levels of cAMP found in caput epididymal sperm relative to those found in caudal epididymal sperm (Hoskins, *et al.*, 1975). Regulation of cAMP levels may be brought about either by Ca^{2+} stimulation of a calmodulin-dependent cAMP phosphodiesterase and/or inhibition of a Ca^{2+}-sensitive adenylate cyclase (Vijayaraghavan and Hoskins, 1990). Wasco and Orr (1984) have reported that Ca^{2+} at concentrations found in caput epididymal sperm stimulates phosphodiesterase activity and it has been shown that bovine sperm contain a calmodulin sensitive adenylate cyclase (Garty *et al.*, 1988). Tash and co-workers (1988) have also reported

the presence of a calmodulin-dependent protein phosphatase in mammalian spermatozoa. Thus Ca^{2+} may regulate sperm motility by controlling the activities of the enzymes involved in cAMP metabolism (Vijayaraghavan and Hoskins, 1990). These workers postulated that the levels of cAMP and hence motility are reduced in caput epididymal spermatozoa due to high intracellular concentrations of Ca^{2+} and as sperm travel along the epididymis the intrasperm levels of Ca^{2+} decrease, probably due to changes in mitochondrial Ca^{2+} handling properties brought about by an increase in intracellular pH, and motility is stimulated.

In some spermatozoa, for example guinea pig and hamster, and in contradiction to the data on bull spermatozoa presented above, one of the actions of Ca^{2+} is the stimulation of cAMP levels. The Mg^{2+}-dependent adenylate cyclases of guinea pig and hamster spermatozoa have been shown to be activated by Ca^{2+} (Hyne and Garbers, 1979a & b). The increase in cAMP levels observed in hamster sperm in response to Ca^{2+} were also associated with an increase in motility (Morton, *et al.*, 1974). It was demonstrated by Garbers and colleagues (1982) that this Ca^{2+} induced elevation of cAMP levels in intact spermatozoa required the presence of bicarbonate. They showed that this Ca^{2+}/bicarbonate induced elevation of cAMP levels was blocked by the addition of D-600, a Ca^{2+} transport antagonist and that the Ca^{2+} ionophore, A23187, which facilitates the movement of Ca^{2+} into a cell, could replace bicarbonate. As sperm contain high quantities of calmodulin, a Ca^{2+} binding protein, (Garbers, *et al.*, 1980) and the activity of some adenyl cyclases is Ca^{2+}/ calmodulin dependent (Brostrum, *et al.*, 1975) it may be that this is one mechanism of activation which operates in these spermatozoa. The anti-calmodulin drug W13 inhibits the motility of spermatozoa, suggesting that the inhibitory effects of Ca^{2+} on flagella motility may be partially mediated through calmodulin (Tash and Means, 1982a & b).

Ca^{2+}-calmodulin complexes can also activate specific protein kinases and other enzymes and via this mechanism Ca^{2+} is thought to be involved in the regulation of

the phosphorylation states of key proteins and enzymes involved in motility (Tash and Means, 1982a & b). Using ejaculated dog sperm lysed with low concentrations of Triton X100 these workers have shown that cAMP increases and Ca^{2+} decreases the incorporation of ^{32}P from [^{32}P]ATP into sperm proteins and this is associated with an increase in motility. This evidence is contradictory to the information stated previously again showing that the evidence for the exact role Ca^{2+} is unclear. Two high molecular weight phosphoproteins, whose phosphorylation states are modified by cAMP and Ca^{2+}, have been identified in these dog sperm models and it has been suggested that one or both of these phosphoproteins may be associated with dynein, the major protein of the flagella responsible for motility (Tash and Means, 1982a & b). The Ca^{2+} induced inhibition of phosphorylation is due to the action of a calmodulin-dependent protein phosphatase which has been identified and localised in the flagella of dog, pig and sea urchin spermatozoa (Tash and Means, 1988). These workers have also shown that this enzyme is associated with dynein as it is found in the dynein fraction when resolved by sucrose gradient centrifugation.

1.4.4. pH.

The spermatozoa of sea urchin remain quiescent whilst in the testes, but upon release into sea water they respire and swim at a maximal rate (Hino, *et al.*, 1980). There are a number of factors which affect respiration and motility in mature spermatozoa including the ionic composition and pH of the media into which they are released and the effect of dilution itself.

The pH of ASW affects respiration and motility of sea urchin spermatozoa (Christen, *et al.*, 1982; 1983). In ASW of normal ionic composition both the motility (Shapiro, *et al.*, 1981; 1990) and respiration of spermatozoa incubated at pH 6.0 are

inhibited when compared with those incubated at pH 8.0 (Ohtake, 1976a & b; Hansborough and Garbers, 1981). Intracellular pH (pHi) can be measured with radioactive or fluorescent amines which are able to transverse membranes and accumulate intracellularly in response to pH gradients (Rottenberg, 1979). Using such techniques it has been shown that quiescent spermatozoa have an acidic pHi (Schackman, *et al.*, 1981; Lee, *et al.*, 1982). The spermatozoa of sea urchin diluted into ONaASW have a more acidic pHi and this is also the case when the external pH is decreased (Christen, *et al.*, 1982), thus indicating a relationship between pHi and motility in spermatozoa. Lee and colleagues (1982) have shown a direct correlation between increased pHi and the activation of respiration and motility of sea urchin spermatozoa. Alkalisation of the pHi of these spermatozoa, by incubation in the presence of NH_4Cl, led to a 50 fold increase in the rate of respiration. Whereas lowering the pH of ASW caused a decrease in pHi of sea urchin spermatozoa and inhibited motility (Hansbrough and Garbers, 1981; Christen, *et al.*, 1982). However, this inhibition was reversed by the addition of agents known to increase pHi i.e. monensin, a Na^+/H^+ exchange ionophore, and speract, a peptide isolated from sea urchin egg jelly (Hansbrough and Garbers, 1981; Repaske and Garbers, 1983).

Na^+-dependent mechanisms are known to regulate pHi in many systems (Nuccitelli and Deamer, 1982) including sea urchin eggs (Lee, 1984a & b). The initiation of motility and respiration of sea urchin spermatozoa upon their release into sea water is also Na^+-dependent, as when these spermatozoa are diluted into Na^+-free sea water (ONaASW) both their respiration and motility are suppressed but this effect is reversed on addition of external Na^+ (Nishioka and Cross, 1978). The activation sea urchin spermatozoa is also accompanied by acid extrusion which again does not occur in the absence of external Na^+ thus implying that Na^+/H^+ exchange may be involved in the initiation of motility in spermatozoa (Nishioka and Cross, 1978). This Na^+-dependent acid extrusion has been shown to lead to an increase in pHi,

which is thought to be the trigger for the initiation of motility in spermatozoa (Christen, *et al.*, 1982; 1983).

Sperm respiration and motility are also sensitive to the K^+ concentration of sea water (ASW). The motility and respiration of sea urchin spermatozoa are inhibited when released into sea water containing high levels of K^+ (Gibbons, 1980) and the pHi of these spermatozoa is more acidic when the extracellular K^+ concentration is increased (Christen, *et al.*, 1982; 1983). A high external K^+ concentration has also been found to inhibit Na^+-dependent H^+ release both in intact sea urchin spermatozoa and in the isolated flagella of these spermatozoa (Lee, *et al.*, 1984a & b). Studies using the membrane potential probe tetraphenylphosphonium (TPP^+) showed that increasing the external K^+ concentration depolarised the membrane potential and caused a decrease in the uptake of TPP^+ both in intact spermatozoa (Schackermann, *et al.*, 1981) and in isolated flagella (Lee, 1984a & b). The possibility that the inhibitory effect of K^+ may be due to the depolarisation of the membrane potential was confirmed by using other methods to induce membrane depolarisation. For example, caesium ions (Cs^+) in the presence of valinomycin, a cation ionophore which transports K^+ and Cs^+ but not Na^+ caused depolarisation of the membrane and inhibited the Na^+/H^+ exchange. However, neither Cs^+ nor valinomycin alone had any effect on either membrane depolarisation or Na^+/H^+ exchange. Thus the dilution of sea urchin spermatozoa into sea water with an elevated K^+ concentration results in the depolarisation of their plasma membrane (Schackerman, *et al.*, 1981; Lee, 1984a & b), which inactivates Na^+/H^+ exchange (Lee, 1984a & b) resulting in the acidification of the pHi and a decrease in motility and respiration (Christen, *et al.*, 1982; 1983).

The activation of sperm respiration is correlated with increased activity of axonemal dynein ATPase (Christen, *et al.*, 1983) which is optimal around pH 7.5 (Gibbons and Gibbons, 1972). Permeabilized sperm have been used to study

axonemal motion over a range of pH. These sperm models are activated over a very narrow pH range (7.3 to 7.8) which correlates closely with the range *in vivo* in which the activation of respiration and motility of sperm occurs (Christen, *et al.*, 1982). There are three possible situations in which an acidic pHi could lead to the inhibition of respiration and consequently the motility of spermatozoa (Christen, *et al.*, 1982; 1983):

I. independent inhibition of both motility and respiration.

II. inhibition of dynein ATPase located in the axoneme which in turn leads to a lack of ADP production thus causing tightly coupled mitochondria to cease respiration.

III. inhibition of mitochondrial respiration resulting in lack of ATP production and cessation of motility.

The dynein ATPase activity of sea urchin spermatozoa is inhibited at an acidic pHi both *in vitro* and *in vivo* and the ATP levels, of these spermatozoa, compared with those observed at an alkaline pHi are increased (Christen *et al.*, 1983). These workers concluded that inhibition of mitochondrial respiration is not a factor in the regulation of sperm motility when pHi is acidic. However, Christen and colleagues (1983) also showed that uncoupled respiration, in the presence of FCCP, is inhibited by acidic pHi. Thus, it is not clear which of the above three mechanisms operate in regulating sperm motility. It is evident from the above that respiration and motility are likely to be linked (Brokaw and Benedict, 1968) and this association could be due to ATP synthesis during respiration being closely coupled to ATP degradation by a single class of enzyme, the dynein ATPase (Christen, *et al.*, 1983).

The pHi of sea urchin spermatozoa is affected by both the Na^+ concentration and pH of the media into which they are diluted thus the alkalisation of pHi required for sperm activation is in part obtained through Na+/H+ countermovements (Lee, *et al.*, 1980; 1982; 1983; Christen, *et al.*, 1982). Protons are continuously produced by metabolism thus a continuous influx of Na^+ is needed in exchange for protons in order

to regulate pHi (Bibring, *et al.*, 1984). This leads to an increase in intracellular Na+ and thus the driving force for H+ efflux decreases. The addition of K^+ (10 mM) to K^+ depleted ASW in which sea urchin sperm are suspended induces an internal alkalisation followed by an influx of K^+. This addition has no effect on the pHi in the absence of external Na^+, in the presence of Na^+ when sperm have been depleted of internal ATP or in the presence of ouabain, a Na^+/K^+ATPase inhibitor. Thus, from these results Gatti and Christen (1985) concluded that a Na+/K+ATPase pump was present in the membrane of sea urchin sperm and that this pump may play a role in the regulation of pHi by recycling the Na^+ that enters the cells through Na+/H+ countermovements.

Mature bovine spermatozoa are stored in the caudal epididymis prior to ejaculation and these spermatozoa, when examined in undiluted caudal fluid (pH 5.8), exhibited minimal movement. When the pH of this fluid was increased to pH 7.0 or the sperm were diluted they became more motile (Acott and Carr, 1984) and this increase in motility was attributed to an elevation in pHi (Babcock, *et al.*, 1983). Carr and Acott (1989) measured the intracellular pH of sperm under physiological conditions using the fluorescent pH probe, carboxyfluorescein, and found that pHi and motility increased when the sperm in caudal fluid were diluted or the pH of the fluid was raised. These authors concluded that bovine sperm remain quiescent whilst in the testes due to the presence of a motility inhibitor in the epididymal fluid and that this factor regulates sperm motility by modulating the pHi of sperm. The ability of certain phosphodiesterase inhibitors, for example theophylline to initiate motility in immature spermatozoa from the caput region of the epididymis may be due not only to their ability to elevate the intrasperm levels of cAMP but also to act as weak bases and increase pHi (Vijayaraghavan, *et al.*, 1985). Therefore, it may be that mature spermatozoa from the caudal region of the epididymis can be stimulated by either a further elevation in pHi or an increase in intrasperm cAMP levels (Vijayaraghavan and

Hoskins 1986) because the pHi of caudal spermatozoa is higher (pH 6.7 ± 0.05) than the pHi of caput spermatozoa (pH 5.84 ± 0.1). Spermatozoa isolated from the caput region of the epididymis require an elevation in pHi before agents which increase cAMP levels have any effect (Majumder, *et al.*, 1990).

As stated previously (Section 1.4.1) cAMP is associated with an increase in sperm motility and relationship between pHi and cAMP-dependent phosphorylation has been reported (Goltz, *et al.*, 1988). Carr and Acott (1989) have identified a protein that is phosphorylated in response to elevations in pHi and three other phosphoproteins have been identified in subcellular sperm fractions. Two plasma membrane phosphoproteins are phosphorylated under conditions which lead to increased pHi and another plasma membrane phosphoprotein is dephosphorylated in response to increasing pHi (Carr and Acott, 1989).

In conclusion, external Ca^{2+} has been observed to have either a stimulatory or an inhibitory effect on motility of sperm depending on the species (Majumder, *et al.*, 1990). The intracellular free Ca^{2+} concentration also affects flagella motility in every species examined (Tash and Means, 1983). The concentration of intracellular Ca^{2+} is regulated by an active Ca^{2+} pump and a Na^{+}/Ca^{2+} exchanger both of which have been shown to be present in the plasma membrane of sperm (Majumder, *et al* 1990). Cyclic AMP has been shown to stimulate motility and respiration of sperm (Garbers, *et al.*, 1971; Hoskins, *et al.*, 1978). Cyclic AMP and Ca^{2+} together may also act as second messengers and regulate sperm motility by the phosphorylation/dephosphorylation of specific proteins required for flagella motility (Majumder, *et al.*, 1990). Ca^{2+} may additionally control motility by bringing about the alkalisation of sperm, though its activation of PKC, which has been shown to phosphorylate and thereby activate Na^{+}/H^{+} exchange in fibroblasts (Stiffert and Akkerman, 1988). Intracellular pH also plays a role in the regulation of sperm motility as agents, for example NH_4Cl, that cause an increase in pHi stimulate both the

motility and respiration of sperm. The addition of certain phosphodiesterase inhibitors, for example theophylline, to immature caput epididymal sperm initiate motility not only because of their ability to raise the intrasperm cAMP level but also to act as weak bases and increase pHi. The motility of mature caudal epididymal sperm can be stimulated by either an increase in pHi or an elevation of intracellular cAMP levels, whereas caput epididymal sperm do not respond to cAMP alone. The reason for this difference may be that these latter sperm have a higher pHi than the caput epididymal sperm (Vijayaraghavan, *et al.*, 1985). An increase in pHi has also been implicated in the activation of sea urchin spermatozoa upon their release into sea water and this alkalisation is thought to be brought about by Na^+/H^+ exchange (Lee, *et al.*, 1983).

The initial aim of this study was to investigate the mechanisms that regulate the initiation of mammalian sperm motility, in particular rat, during sperm maturation. The intention was to ascertain which ion pumps/channels were present in the plasma membrane of sperm and to examine their role in the regulation of sperm motility. Unfortunately, approximately one year into the project a problem arose in that the spermatozoa of the rats supplied were becoming totally immotile within a few minutes of diluting them into the incubation medium. A great deal of effort was expended in trying to establish whether the media or the rats themselves were the source of this problem. For example the batch number of the media was checked to see that it was the same as the media previously used and it was. Then we tried the same type of media but from a different supplier, again to no avail, so we next tried diluting the spermatozoa into phosphate buffer however, the spermatozoa still become immotile within a few minutes of dilution. Finally we purchased rats from different suppliers unfortunately the spermatozoa were still immotile within a few minutes after dilution. At the time the concentration of sperm used was $5x10^8$. More recently it has become

apparent (Fraser, 1994) that the motility of rat sperm is highly concentration dependent and that there is considerable inter animal variation in this dependence. It is now clear that optimal concentrations for motility are within the range $5x10^6$ to $1x10^7$ and hence this could explain the problems which were encountered.

Whilst we were trying to sort out this problem Dr Paul Bolwell, who had previously worked on the fertilisation of *Fucus serratus*, suggested that I look at the mechanisms involved in the activation of sperm from *Fucus serratus* upon their release into sea water. The objectives of this study were:-

I. To examine whether the ions present in sea water influenced sperm motility and respiration.

II. To determine the presence of ion pumps/channels and examine the role they play in the activation of these sperm.

III. To examine the role of second messengers in the initiation and regulation of motility of sperm from *Fucus serratus*.

CHAPTER 2

Materials and Methods.

2. MATERIALS AND METHODS.

2.1. Isolation of Mammalian Spermatozoa.

Adult male Sprague-Dawley and Wistar rats were killed by cervical dislocation and their epididymides were quickly removed and separated into caput and caudal regions. These organs were transferred to moist filter paper where adherent adipose tissue was removed and then placed into sterile Petri dishes containing 10 cm^3 Earles' medium (Flow Laboratories, Irvine, UK) supplemented with 4 mg/cm^3 bovine serum albumin and each 100 cm^3 of media was supplemented with 100 U/cm^3 penicillin and 0.1 mg/cm^3 streptomycin. Caput and caudal epididymides were punctured several times with a 26-gauge needle and the sperm were released by gentle compression of the epididymides using broad tipped forceps. The released spermatozoa were pelleted by centrifugation at 500g for 5 minutes, at room temperature, followed by 1200g for 10 minutes. The pelleted spermatozoa were resuspended in the media ± bicarbonate at concentrations described in the results section. A 10 µl sample was removed for counting using an improved Neubauer haemocytometer. The concentration of spermatozoa was then adjusted to 5 x 10^8 spermatozoa/cm^3 and the accuracy of this adjustment was checked by determining the cell concentration after dilution.

2.2. Isolation of Spermatozoa from *Fucus serratus*.

Male *Fucus serratus* plants were obtained by staff at the Marine Biological Station at Millport, Isle of Cumbie, Scotland and delivered within 24 hours of collection. Plants were stored between wet newspaper at 10°C and used within a week of collection for all experiments. Spermatozoa were obtained by gently washing

the thallus of the plants with either artificial sea water (ASW) or modified ASW as described in the results section, using a Pasteur pipette.

2.3. Composition of Artificial Sea water.

ASW was of the following composition:

360 mM NaCl

50 mM $MgCl_2$

10 mM $CaCl_2$

10 mM KCl

30 mM Hepes. pH 8.0 (Christen *et al.*, 1982).

Periodically the osmolality of the ASW was checked using a osometer. Readings were in the range of 960-1050 mOsm/litre.

In some experiments modified ASW was used. For experiments requiring Na^+-free sea water (ONaASW) sodium chloride was replaced by N-methylglucamine (360 mM) (Sigma, UK) which was prepared as a 1M solution and titrated to pH 8.0 with concentrated HCl (Brawley and Bell, 1987). When the concentration of K^+ was varied, Na^+ was substituted for K^+, so that $[K^+] + [Na^+]$ was always 370 mM. Ca^{2+}-free ASW (OCaASW) was made by omitting Ca^{2+} from ASW, adding 1 mM EGTA and the pH was readjusted. In each case the osomolality was checked and adjusted were necessary.

2.4 Activators and Inhibitors.

The activators/inhibitors used in this study were diluted in the following solvents:-

Amiloride	Dimethylsulfoxide (DMSO)	SITS	H_2O
Dibutyl cAMP	H_2O	Theophylline	DMSO
Forskolin	DMSO	Vanadate	DMSO
Monensin	Ethanol	Verapamil	DMSO
Ouabain	DMSO		

Each of the above agents were made up at 100 times the required concentration in the appropriate solvent so when added to suspensions of either mammalian or *Fucus serratus* spermatozoa the final concentration of solvent was 1%. Control suspensions of spermatozoa contained the appropriate concentration of solvent.

Mammalian spermatozoa from both regions of the epididymis (5×10^8 spermatozoa/cm^3) were incubated at 37°C in medium for between 30-60 minutes, as indicated in individual experiments, in the presence or absence of activators/inhibitors. Spermatozoa from *Fucus serratus* diluted in the appropriate ASW were incubated at 10°C for 10 minutes, again in the presence or absence of activators/inhibitors.

In studies on spermatozoa of *Fucus serratus* each replicate experiment was carried out using different batches of seaweed.

2.5. Motility Measurement.

At the end of each experiment with either mammalian or *Fucus serratus* spermatozoa 10 µl aliquots were removed using a capillary pipette, placed on a haemocytometer and assessed for motility.

Motility was determined by two methods:

1) visual evaluation using a phase-contrast microscope,

2) by the video microscopic procedure of Katz and Overstreet (1981). Motility was recorded on videotape using a Panasonic video camera and a 20 x phase objective. Videotape records were made and analysed on a Panasonic video cassette recorder using freeze framing and variable speed framing. The percentage of motile spermatozoa was determined by counting all spermatozoa in a video field and dividing the number which were motile by the total present. For each experiment the percentage motility was calculated for at least five video fields and the results averaged.

2.6. Measurement of Sperm Respiration.

Respiration rates were determined by continuous recording with a Clarke type oxygen electrode which consists of a platinum cathode and silver anode in saturated potassium chloride solution. When a potential is applied across the "cell" formed by these electrodes dipping into a test solution four electrons generated at the anode reduce a molecule of oxygen at the cathode. If the polarising voltage is in the range 0.5-0.8 V, then the current generated is directly proportional to the concentration of oxygen in the test solution and the change of oxygen concentration with time can be recorded.

To calibrate the electrode zero oxygen concentration was obtained by adding a few crystals of sodium dithionite to the buffer solution and adjusting the pen of the chart recorder to zero. Air-saturated ASW was taken to be 100% oxygen and the pen on the chart recorder adjusted accordingly. The solubility of oxygen in this aqueous solution at 10°C is 282.01 µmol of dissolved oxygen per litre (Benson and Krause. Jr. 1984). In these experiments the closed vessel of the oxygen electrode contained a reaction volume of 4 cm^3. Thus the saturated oxygen content in the reaction mixture was 4 x 282.01 = 1128.04 nmol. The full scale deflection on the chart recorder was adjusted to 96 divisions for the O_2 saturated ASW therefore each division on the chart was equivalent to 11.75 nmol of O_2.

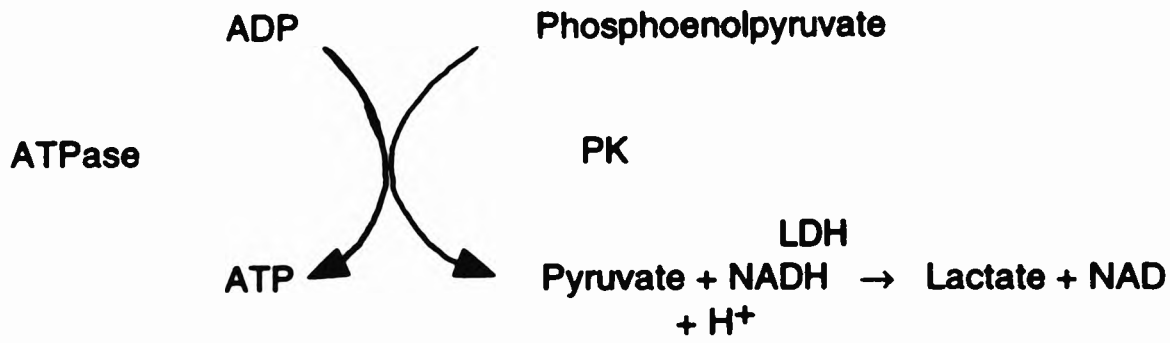
Sperm respiration was measured by adding 4 cm^3 of suspended *Fucus serratus* spermatozoa ($1x10^8$ spermatozoa/cm^3) to the reaction vessel of the O_2 electrode. Oxygen uptake of the continuously stirred suspension was monitored at 10°C using the calibrated chart recorder. In some experiments suspensions of spermatozoa were incubated at 10°C for 10-15 minutes in the presence of a test reagent before measuring O_2 uptake. Where the experiment was to investigate whether these reagents had an immediate effect on O_2 uptake, then additions from 10-100 x stock solutions were made through a small hole in the top of the closed reaction vessel. All experiments were carried out with some light but this was minimised by turning off all artifical lights and partial blocking out daylight on bright days.

2.7. *In vitro* ATPase Assay.

The ATPase activity of spermatozoa from *Fucus serratus* was estimated using the coupled assay of Gibbons and colleagues (1978), in which the ADP formed by ATP hydrolysis was coupled with Phosphoenolpyruvate to form pyruvate in the presence of Pyruvate kinase, which in turn oxidised NADH, in the presence of lactate

dehydrogenase, as shown in Figure 2.I. This reduction of NADH could be monitored on a spectrophotometer, by a decrease in absorbance at 340nm.

Figure 2.I. Schematic representation of the coupled ATPase assay.



PK = Pyruvate kinase
LDH = Lactate dehydrogenase

An aliquot of sperm suspension (10 µl), containing 1-2x10^8 sperm/cm^3, was diluted into 0.4 cm^3 of a solution containing 150 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes, 1 mM EDTA and 0.04% (v/v) Triton X-100; pH 6.6, and mixed for 20 seconds by tapping the base of the tube by hand. Twenty µl of this permeabilized sperm suspension was transferred to a tube containing 1 cm^3 of reaction mixture composed of 150 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes, 1 mM EDTA, 1 mM dithiothreitol, 40 µg/cm^3 of pyruvate kinase (16 units/cm^3), 24 µg/cm^3 of lactate dehydrogenase (22 units/cm^3), 100 µM NADH, 0.5 mM ATP and 1.5 mM phosphoenolpyruvate at varying pH (7.0 → 8.4). Activity was followed as the decrease in absorbance at 340nm. To investigate which ATPase, if any, was responsible for the activity, various activators/inhibitors of individual ATPases were added to the reaction mixture from 10 x stock solutions to give the following final concentrations: 1 µM oligomycin, 10 µM vanadate (Calicobiochem, Nottingham UK) and 1 mM ouabain.

2.8. Isolation of Sperm Flagella from *Fucus serratus*.

Ripe spermatozoa were washed off the thallus of male *Fucus serratus* plants with ONaASW and the concentration of spermatozoa adjusted to 1 x 10^8

spermatozoa/cm^3. The lack of Na^+ prevents the activation of motility. Spermatozoa were pelleted by centrifugation at 600 x g for 5 minutes. 200µl aliquots of the loose pellet were diluted with 50 cm^3 of cold ONaASW and homogenised in an ice bath using a Potter-Elvehgem glass homogenizer fitted with a glass pestle. About 25-30 strokes were enough to detach most of the flagella, as monitored by phase-contrast microscopy. The homogenate was centrifuged at 4°C for 30 minutes at 4000g to pellet the sperm heads and any remaining intact spermatozoa. The supernatant fluid containing the detached flagella was centrifuged at 12000g for 30 minutes and the flagella pellet was resuspended in 1-2 cm^3 of ice cold ONaASW. Phase-contrast microscopy was used routinely to monitor the extent of contamination by sperm heads or intact sperm in the final flagella suspension.

2.9. Measurement of Protein Concentration.

The protein concentration of spermatozoa was estimated by the Lowry (1951) method. Protein standards were made from a stock solution of bovine serum albumin (200 µg/cm^3).

The following stock solutions were prepared:

A. 2% w/v Na_2CO_3 in 0.1 M NaOH

B. 1% w/v $CuSO_4$

C. 2% w/v potassium sodium tartrate

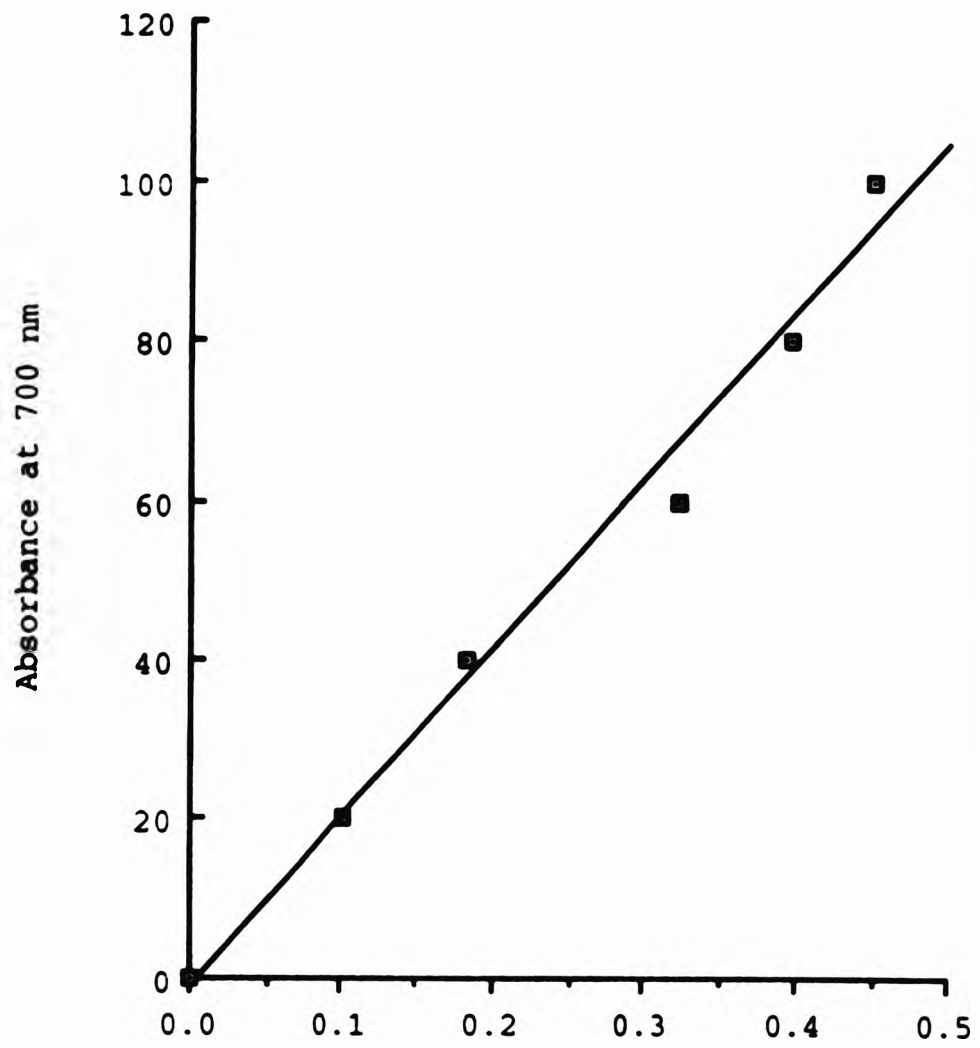
D. Folin ciocalteau phenol reagent was diluted 1 in 3.

Just before use 1 cm^3 of reagent B was mixed with 1 cm^3 of reagent C and added to 100 cm^3 of reagent A to form an alkaline copper reagent.

To each duplicate standard (0.5 cm^3), 2.5 cm^3 of the alkaline copper reagent was added, mixed well and left at room temperature for 10 minutes. Diluted Folin ciocalteau phenol reagent (0.25 cm^3) was added to each standard, mixed well and left at room temperature for 30 minutes. The absorbance of each standard was read at 700 nm and plotted against the concentration of bovine serum albumin to give a standard curve (Figure 2.II).

Duplicate samples (100 µl) of sperm suspension were diluted to 0.5 cm^3 with distilled water and treated as for the standards. The absorbance of the samples as read at 700 nm and the concentration of protein deduced from the standard curve.

Fig. 2.I. Represenative Protein Standard Curve



2.10. Measurement of the Efflux of H^+.

H^+ release by both intact spermatozoa and the flagella was monitored using a pH electrode connected to a digital pH/ion meter (Corning 135). Both intact spermatozoa and isolated flagella were suspended in unbuffered ONaASW to a final protein concentration between 100 and 200 $\mu g/cm^3$ and the pH adjusted to 8. The suspension (3 cm^3) was continuously mixed with a magnetic bar. To increase the sensitivity of H^+ measurement Hepes was omitted from the ONaASW.

2.11. Measurement of Intracellular pH.

The intracellular pH (pHi) of spermatozoa from *Fucus serratus* was determined using the pH-sensitive fluorescence indicator dye 2',7'-bis-(-2-carboxyethyl)-5(and-6)carboxyfluoresceinacetoxymethyl ester (BCECF-AM) (Caliobiochem, Nottingham, UK).

Spermatozoa loaded with BCECF-AM were prepared as follows:
Spermatozoa suspensions ($1x10^8$ spermatozoa/cm^3 ASW) were incubated for 2-3 hours with 5 µM BCECF-AM, which was added from a 1 mM stock solution in DMSO. Suspensions were then centrifuged at 600g, at room temperature, for 10 min, washed three times with ASW and finally resuspended in 3 cm^3 ASW. The suspension (1 cm^3) was transferred to a 1 cm path length cuvette and fluorescence intensity was scanned over the excitation range 400 → 550 nm in a Perkin-Elmer LS50 fluorescence spectrometer. Spectra were obtained for sperm suspensions before and after loading with BCECF-AM. Excitation spectra of the free acid, BCECF as well as its acetoxymethylester, BCECF-AM (final concentrations: 5 µM) were also obtained in ASW.

The intracellular hydrolysis of BCECF-AM, was measured in intact, heat-treated or lysed cells. Suspensions of spermatozoa ($1x10^8$ spermatozoa/cm^3 ASW) were heat treated at 80°C for 30 minutes. Lysed spermatozoa were obtained by the addition of Triton X-100 [0.1% (w/v)] and gentle shaking for approximately 10 minutes. Both intact and treated suspensions of spermatozoa were loaded with BCECF-AM (final concentration 5 μM), as described above, samples were removed at regular intervals over a period of 3 hours and fluorescence measured (excitation 465 nm; emission 550 nm).

In order to measure the intracellular pH of spermatozoa, fluorescence was measured following excitation at two different wavelengths, before and after treatment with the detergent, digitonin. The differences in the fluorescence intensity ratios following excitation at the two wavelengths is pH-dependent. Suspensions of spermatozoa, loaded with BCECF-AM as previously described, were resuspended in 3 cm^3 ASW with pH values ranging from 6.0 → 10.0. Fluorescence intensity was measured using excitation wavelengths of 490 nm and 465 nm, (emission was set at 550 nm), before and after the addition of 100 μg/cm^3 digitonin (prepared as a 100x stock solution in DMSO). The differences in the fluorescence intensity ratios were plotted as a function of external pH. The value of the internal pH of the spermatozoa was obtained by a interpolation to fluorescence ratio difference of zero.

2.12. Location of Intracellular Ca^{2+}.

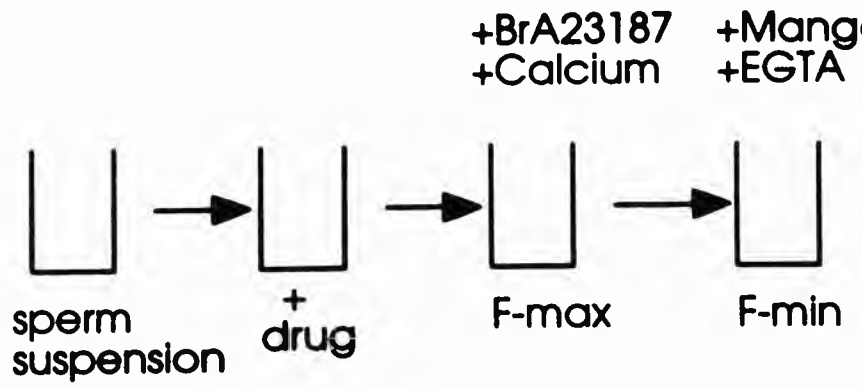
Intracellular Ca^{2+} was located in the spermatozoa of *Fucus serratus* using a zenth standard epifluorescence microscope equipped with filters suitable for the measurement of Fura-2 (350 nm and 385 nm). Photographs were taken with a camera loaded with a T max. 400 film.

2.13. Measurement of the Intracellular Ca^{2+} Concentration.

The determination of intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) of spermatozoa was carried out with the fluorescent indicator Fura-2 (Molecular Probes Inc., Eugene, Oregon, USA), using a modified method of Grynkiewicz *et al.*, (1985). Stock solutions of Fura-2 acetoxymethylester (Fura-2-AM) were 1 mM in DMSO and stored desiccated at -20°C until use. To aid the uptake of Fura-2-AM, 3 µl Pluoronic F-127 (Molecular Probes Inc., Eugene, Oregon, USA), (0.25 mg in 75 µl DMSO heated at 40°C for 20 minutes), was added to 2 µl of Fura-2-AM. This mixture was added to 0.5 cm^3 OCaASW and the resultant solution was sonicated for 30 seconds on ice before addition of 0.5 cm^3 of sperm suspension. The incubating solution contained final concentrations of 2 µM Fura-2-AM and $1x10^8$ spermatozoa/cm^3. After incubation for 3 hours at 4°C the spermatozoa were pelleted by centrifugation at 600g, at 4°C, for 5 min and then washed three times to minimise the carry-over of extracellular Fura-2-AM. The sperm pellet was finally resuspended in 1 cm^3 OCaASW and transferred to 1 cm path-length quartz cuvettes. Fluorescence intensity was measured in a Perkin Elmer LS50 fluorescence spectrophotometer. For the examination of fluorescence spectra, the emission wavelength was set at 500 nm, and the excitation wavelength was scanned over the range 300 → 500 nm, with an emission slit of 10 nm and an excitation slit of 1.5 nm. Spectra were obtained for sperm suspensions before and after loading with Fura-2-AM.

Figure 2.III shows a diagrammatic representation of a typical experiment to measure the intracellular calcium concentration in the presence and absence of activators or inhibitors of motility in spermatozoa of *Fucus serratus*.

Figure 2.III. Diagrammatic representation of a typical experiment to measure the $[Ca^{2+}]i$ of spermatozoa of *Fucus serratus*.



Internal free calcium was calculated from the equation of Grynkiewicz and colleagues (1985):

$$[Ca^{2+}]i = Kd \times (R - R_{min}) / (R_{max} - R)$$

Kd = the dissociation constant for Fura-2-Ca^{2+} and taken to be 220 nM (Rink and Rozzan, 1985).

R = the ratio of fluorescence intensity at excitation wavelengths of 350 and 385 nm with emission at 500 nm. The fluorescence intensity at this ratio is Ca^{2+}-dependent (Grynkiewicz, *et al.*, 1985). Rmin and Rmax are the ratios of the fluorescence intensities obtained at excitation wavelengths of 350 and 385 nm, (emission was set at 500 nm), under Ca^{2+} free and Ca^{2+} saturating conditions respectively.

Maximum fluorescence (F_{max}) was determined following excitation at the two wavelengths separately in the presence of the calcium ionophore Bromo A23187 (final concentration: 25 μM) and Ca^{2+} (final concentration: 10 μM). Minimum fluorescence (F_{min}) was determined at the two excitation wavelengths in the presence of 4 mM EGTA at pH 8.0 and manganese (final concentration: 10 μM), which quenches Ca^{2+}-dependent fluorescence. Figure 2.IV shows typical scans for control, F_{min} and F_{max}.

The intracellular free Ca^{2+} concentration was also measured in the presence of activators and inhibitors of sperm motility. Suspensions of spermatozoa ($1x10^8$ spermatozoa/cm^3) containing known concentrations of either an activator or inhibitor of sperm motility added from 100x stock solutions were incubated for 15 minutes at 10°C, these suspensions were then centrifuged at 600g and the pellet resuspended in 3 cm^3 OCaASW and loaded with Fura-2-AM as previously described.

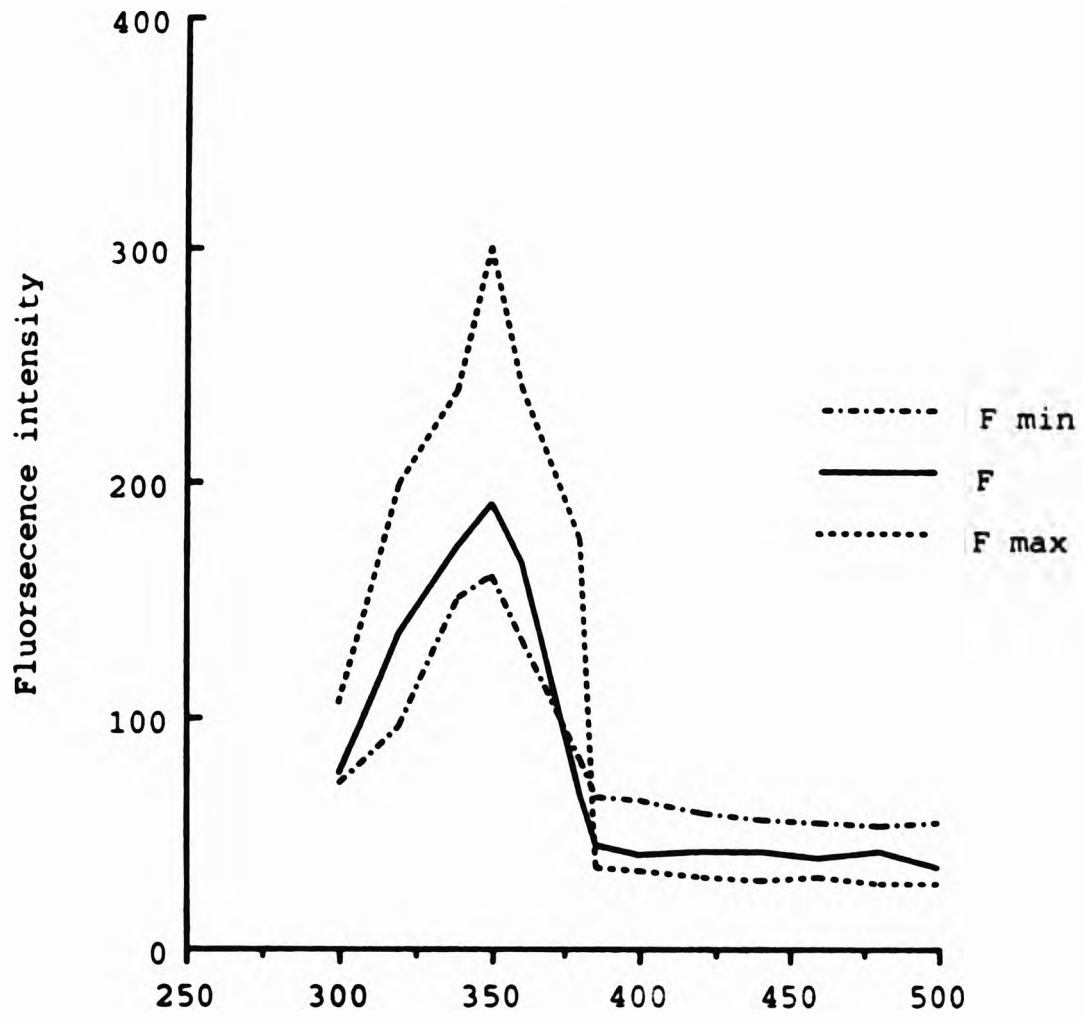
2.14. Chemicals.

All the chemicals used, unless stated, were obtained from either Sigma Chemical Co. (Poole, Dorest, UK) or Merck BDH (Poole, Dorest, UK), and were of analytical grade.

2.15. Statistical Analysis.

Statistical comparisons were performed using Student's t test. Unless stated all test were compared with untreated controls.

Figure 2.IV. Typical Excitation Spectra for Spermatozoa suspended in OCaASW.



Typical excitation spectra at 500 nm emission for *Fucus serratus* sperm suspension incubated in OCaASW. The intracellular free Ca^{2+} concentration was calculated as described above.
F_{min} = + EGTA & manganese
F = Spermatozoa + Fura-2-AM only
F_{max} = + Bromo A23187 & Ca^{2+}.

RESULTS

CHAPTER 3

Activation of Mammalian Spermatozoa

3. Mammalian Spermatozoa.

Mammalian spermatozoa acquire the capacity for motility during transit through the epididymis (Bedford, 1975). Spermatozoa, isolated from the caput epididymis are virtually immotile, although some may display a twitching motion whereas spermatozoa isolated from the caudal region of the epididymis are motile with approximately 10% showing vigorous forward motion (Hoskins, *et al* ., 1978; Vijayaraghavan, *et al.*, 1985). The biochemical basis for this acquisition of the capacity for motility is not fully understood.

In the present study the percentage of rat spermatozoa, isolated from the caput region of the epididymis, which were motile was only 2.2 ± 1.7% (n = 6) whereas 28.7 ± 3.7% (n = 6) of spermatozoa isolated from the caudal region of the epididymis were motile, with 17.3 ± 2.9% of these spermatozoa displaying fast forward progressive motility.

3.1. Factors Affecting the Acquisition of Motility During Epididymal Transit.

A number of factors have been reported to affect the motility of mammalian spermatozoa and these include a number of second messengers. Of these particular attention has been placed on pH both external and internal, cAMP and calcium.

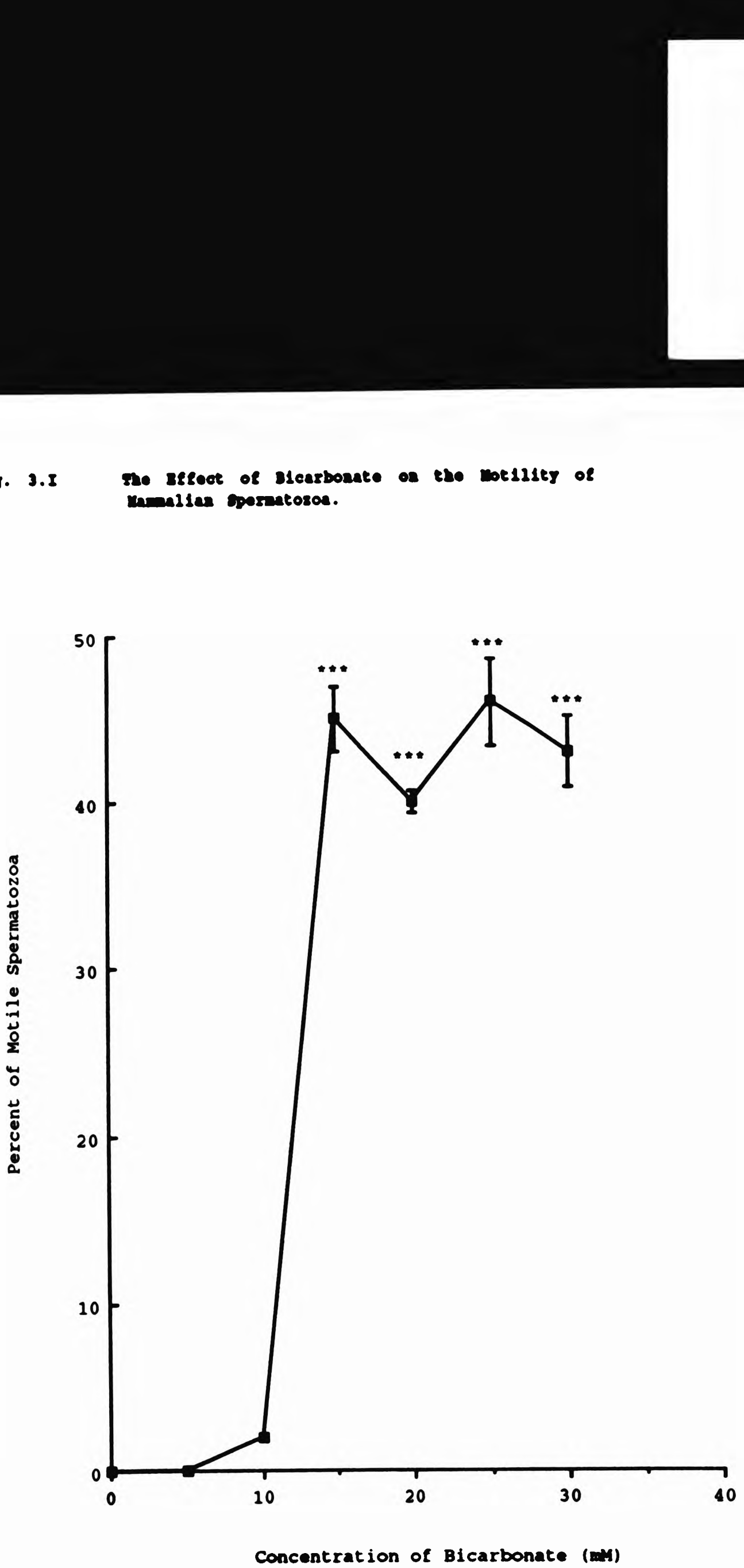
3.1.1. pH.

Following the observation that bicarbonate dramatically stimulates the motility levels of guinea pig spermatozoa (Garbers, *et al.*, 1983) and bovine spermatozoa (Vijayaraghavan, *et al.*, 1985), the effect of bicarbonate on the motility of rat caput epididymal spermatozoa was tested. The bicarbonate ion was found to have a

stimulatory effect on the motility of these spermatozoa. The results in Figure 3.I show that there is an increase in motility of caput epididymal spermatozoa from 0.0 ± 0.5% to 45 ± 3.9% with increasing bicarbonate concentrations up to 15 mM. No further stimulation of motility was observed when the concentrations of bicarbonate were increased from 15 mM to 30 mM.

In the case of spermatozoa from the caudal region of the epididymis the addition of bicarbonate at the optimum concentration of 15 mM also enhanced the number of motile spermatozoa by 196% from 28.7 ± 3.7 to 85.0 ± 3.0%.

Fig. 3.I The Effect of Bicarbonate on the Motility of Mammalian Spermatozoa.



Washed spermatozoa isolated from the caput region of the epididymis (1-2x10^8 spermatozoa) were incubated with increasing concentrations of bicarbonate at 37°C for 30 minutes. The percent of motile sperm was measured as described in the Materials and Methods section (n=10).
*** P< 0.001.

Other studies have shown that an increase in intracellular pH stimulates motility and respiration in bovine caudal sperm (Babcock, *et al.*, 1983) and is involved in the activation of sea urchin spermatozoa (Lee, *et al.*, 1983). Vijayaraghavan and colleagues (1985) showed that the presence of bicarbonate in the incubating media leads to an elevation of intracellular pH.

Bicarbonate enters a cell through a bicarbonate/chloride exchanger which acts by exchanging external Na^+ and HCO_3^- for internal H^+ and Cl^-. The HCO_3^-/Cl^- exchanger is involved in the regulation of intracellular pH in mammalian cells. for example, it accounts for 20% of the active acid extrusion from mouse soleus muscle (L'Allemain. *et al.*, 1985). This exchanger is inhibited by stilbene derivatives such as 4-acetoamido-4`-isothiocyanostilbene-2,2`-disulfinic acid (SITS) (L'Allemain, *et al.*, 1985).

Spermatozoa isolated from both regions of the rat epididymis were incubated in medium containing bicarbonate in the presence of varying concentrations of SITS for 30 minutes, after which samples were removed and motility observed microscopically (Table 3.I).

Table 3.I. The effect of SITS on the motility of spermatozoa isolated from the caudal region of the epididymis

Concentration of SITS mM	Percent of Motile Spermatozoa CAPUT	CAUDAL
0	45.0 ± 2.5	78.0 ± 3.7
0.1	40.0 ± 1.5	70.0 ± 1.8
0.5	33.0 ± 2.0	52.0 ± 2.2 ***
1	18.0 ± 1.2 ***	22.5 ± 1.7 ***

Values are means ± SD (n=6).
*** P<0.001.

The results show that at 1 mM, SITS inhibited the motility of spermatozoa from both the caput and caudal regions of the epididymis by 60 and 70% respectively. Thus it would appear that the presence of bicarbonate plays a significant role in the regulation of motility.

Another exchanger which extrudes H^+ from inside the cell and thus leads to internal alkalisation is the Na^+/H^+ exchanger. Monensin, an activator of the Na^+/H^+ exchanger (Pressman, 1976), caused activation of rat spermatozoa from both caput and caudal regions (Table 3.II).

Table 3.II. The effect of monensin on the motility of spermatozoa.

Epididymal origin of sperm	Concentration of Monensin mM	Percent of Motile Spermatozoa
Caput	0	37.0 ± 1.5
	0.1	52.0 ± 1.8
	1	66.0 ± 2.5
	10	89.0 ± 3.6 ***
Caudal	0	77.0 ± 3.7
	0.1	80.0 ± 1.8
	1	88.0 ± 3.6
	10	95.0 ± 4.0 ***

Values are means ± SD (n=6).
*** P<0.001.

Incubation of both populations of epididymal spermatozoa with 10 mM monensin for 30 minutes enhanced their motility. However the extent of stimulation of the two populations differed. The motility of caput spermatozoa was increased by 157% whereas the motility of caudal epididymal sperm were only increased by 23%.

Thus it would appear that agents which affect the intracellular pH of rat spermatozoa also regulate their motility but their effect is greater in sperm from caput

than in those from the caudal epididymal regions suggesting that the pH in spermatozoa from the caudal region may already be closer to that required for maximum motility.

3.1.2. Adenosine 3`.5`-cyclic Monophosphate.

Adenosine 3`,5`-cyclic monophosphate (cAMP) has also been implicated in the acquisition of the capacity for motility which occurs as sperm travel through the epididymis (Hoskins, *et al.*, 1974). Theophylline is an agent known to elevate cAMP levels. From Table 3.III it can be seen that the addition of this agent to suspensions of spermatozoa increased the number of motile sperm and addition of bicarbonate further enhanced the number of motile sperm obtained from the caput region which were observed.

Table 3.III. The effect of theophylline on the motility of spermatozoa.

Epididymal origin of sperm	Additions	Percent of Motile Spermatozoa
Caput	None	2.0 ± 1.8
	Bicarbonate (15mM)	45.0 ± 3.9 ***
	Theophylline (1mM)	11.0 ± 4.5 **
	Bicarbonate (15mM) + Theophylline (1mM)	68.0 ± 4.8 ***
Caudal	None	28.0 ± 3.7
	Bicarbonate (15mM)	85.0 ± 3.0 ***
	Theophylline (1mM)	84.0 ± 2.5 ***
	Bicarbonate (15mM) + Theophylline (1mM)	87.0 ± 4.5 ***

Values are means ± SD (n=10).
** P<0.01 *** P<0.001.

The addition of theophylline or bicarbonate alone to spermatozoa from the caput region of the epididymis, stimulated motility by approximately 450% and 1250% respectively. However when both these agents were added to caput spermatozoa sperm motility increased by approximately 2400%. The motility of caudal spermatozoa is stimulated by 200% by theophylline, a level comparable to that seen when sperm are incubated in the presence of bicarbonate. However, in contrast to caput sperm, caudal sperm incubated in the presence of both bicarbonate and theophylline showed no further increase in motility.

The effect of theophylline with or without bicarbonate on caput sperm was examined at different concentrations of this agent and the results are presented in Figure 3.II. The data show that in the absence of bicarbonate the percent of motile sperm increases from 2.0 ± 1.5 to 48 ± 3.5% as the concentration of theophylline is increased from 0 → 20 mM. On the other hand in the presence of bicarbonate a dramatic increase in motility is observed at only 1 - 2 mM theophylline and the levels of motility achieved are greater than those observed in the presence of 20 mM theophylline alone.

Forskolin, a diterpene, is also known to elevate cAMP levels in other tissues (Seamon and Daly, 1981a & b) through activation of adenylate cyclase.

From Table 3.IV it can be seen that forskolin stimulates the motility of sperm isolated from the caudal regions of the epididymis. Forskolin has no significant effect on the motility of caput sperm in the absence of bicarbonate however, the presence of bicarbonate stimulated the motility of these sperm by 173%.

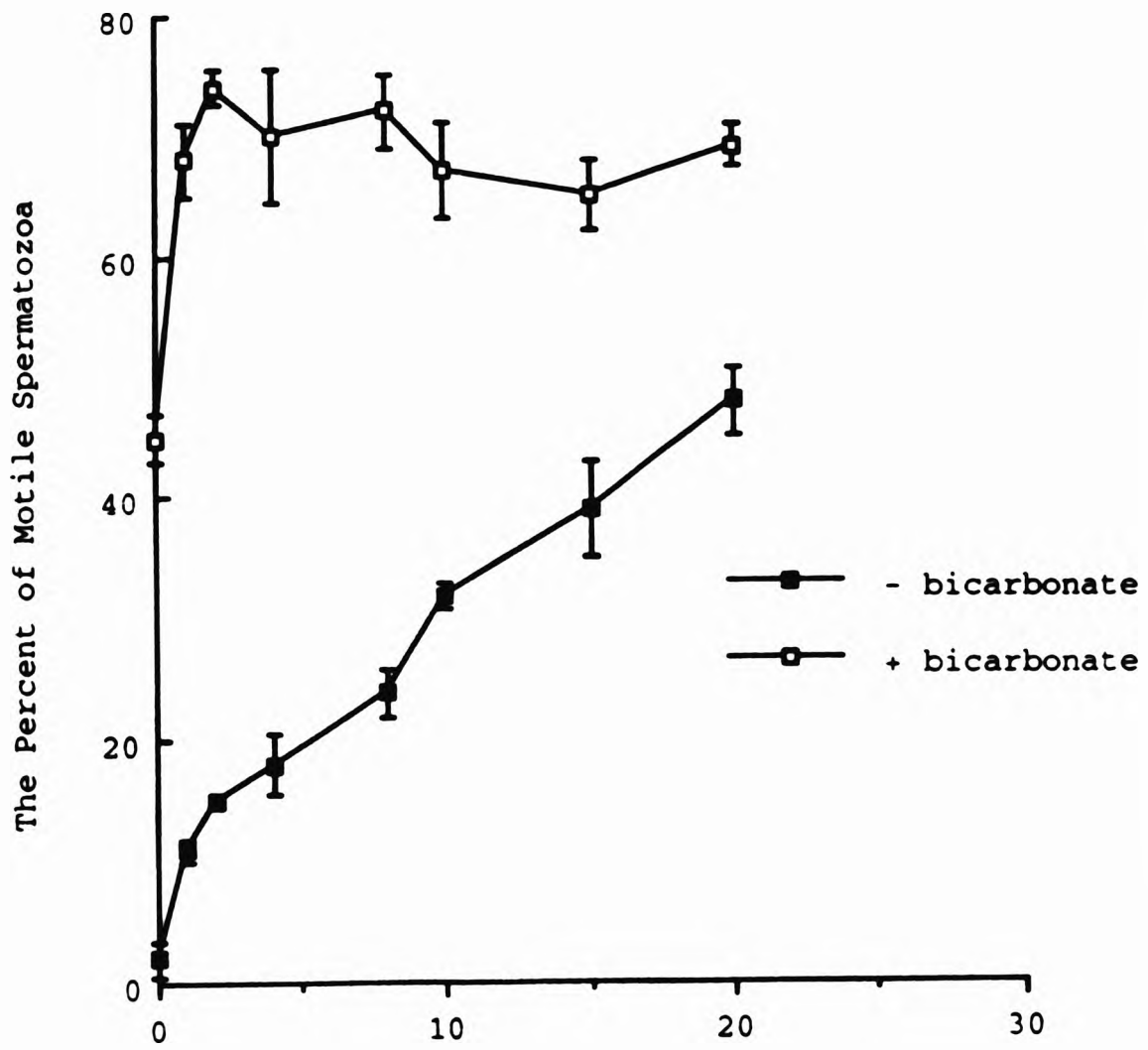
Table 3.IV. The effect of forskolin on the motility of spermatozoa.

Concentration of Forskolin µM	Percent of Motile Spermatozoa			
	CAP (+)	CAP (-)	CAUD (+)	CAUD (-)
0	45.0 ± 2.3	3.0 ± 1.7	80.0 ± 3.0	28.7 ± 3.7
0.1	58.5 ± 1.8***	3.1 ± 1.5	88.0 ± 1.5**	30.0 ± 0.6
1	65.0 ± 2.0***	3.6 ± 0.5	96.5 ± 0.5***	35.5 ± 1.0**
10	78.0 ± 3.9***	4.2 ± 0.6	99.0 ± 0.5***	40.6 ± 2.0***

Values are means ± SD (n=6).
CAP = Caput epididymal spermatozoa + = + bicarbonate (15 mM)
CAUD = Caudal epididymal spermatozoa - = no bicarbonate
** P<0.01
*** P<0.001.

Thus it can be seen that in the presence of forskolin the motility of caput epididymal spermatozoa was once again enhanced in the presence of bicarbonate. The addition of forskolin to sperm isolated from the caudal region of rat epididymides stimulated motility both in the absence and presence of bicarbonate. These results suggest that further stimulation of motility in the mature sperm can be achieved by either the addition of bicarbonate or forskolin, whereas the immature sperm isolated from the caput region of the epididymis requires both bicarbonate and forskolin to acquire maximal motility.

Fig. 3.II. The Effect of Theophylline on the Motility of Mammalian Spermatozoa.



Washed spermatozoa isolated from the caput region of the epididymis (1-2x10^8 spermatozoa) were incubated with increasing concentrations of theophylline at 37°C for 30 minutes in the presence or absence of bicarbonate. The percent of motile sperm was measured as described in the Materials and Methods section (n=6).

3.1.3. Calcium.

Results obtained, from studies using membrane permeabilized sperm from both mammalian and invertebrate sources, on the role of external Ca^{2+} in modulating the motility of intact sperm are conflicting. For example, external Ca^{2+} stimulates hamster sperm motility but inhibits the motility of sperm from guinea pig, ram and mouse (Vijayaraghavan and Hoskins, 1989).

Table 3.V shows the motility of rat spermatozoa isolated from the epididymis which had been suspended in media, both in the presence and absence of bicarbonate, containing varying concentrations of verapamil a known inhibitor of voltage-dependent Ca^{2+} channels. Sperm were incubated with verapamil for 30 minutes at 37°C after which time samples were removed and motility estimated microscopically.

Table 3.V. The effect of verapamil on the motility of spermatozoa.

Concentration of verapamil µM	Percent of Motile Spermatozoa			
	CAP (+)	CAP (-)	CAUD (+)	CAUD (-)
0	42.0 ± 1.7	2.0 ± 1.5	85.0 ± 3.0	27.0 ± 2.5
0.1	31.0 ± 1.0	0	76.0 ± 1.8	20.5 ± 1.8
10	25.0 ± 2.0	0	67.0 ± 2.0**	16.8 ± 2.6**
100	17.0 ± 3.5 ***	0	55.0 ± 1.5***	10.0 ± 2.0***

Values are means ± SD (n=6).
CAP = Caput epididymal sperm (+) = + bicarbonate (15 mM)
CAUD = Caudal epididymal sperm (-) = no bicarbonate
** P<0.01
*** P<0.001.

The results show that verapamil significantly inhibits the motility of spermatozoa isolated from the caudal region of the epididymis compared with control spermatozoa, which had been incubated over the same period of time without any additions. However, higher concentrations of verapamil are required to inhibit motility when bicarbonate is present. The results with caput spermatozoa also show that verapamil inhibits their motility and like mature spermatozoa slightly higher concentrations of verapamil are required to inhibit motility in the presence of bicarbonate. This suggests that the entry of Ca^{2+} though voltage-dependent channels could be regulated by intracellular pH.

One way Ca^{2+} may be involved in the activation of sperm motility is through the action of Ca^{+}-dependent protein kinase C which is activated by diacylglycerol, a second messenger formed when phosphatidylinositol-4,5-bisphosphate (PIP_2) is hydrolysed by phospholipase C (De Jonge, *et al.*, 1991). Protein kinase C (PKC), like other kinases, phosphorylates a number of intracellular proteins. Therefore, if this PIP_2 system is involved in the acquisition of motility in spermatozoa, then agents that affect the activity of PKC might be expected to alter sperm motility.

Phorbol diesters are a class of tumour-promoting agents which bind with high affinity and selectivity to PKC (MacEwan, *et al.*, 1993). In order to determine if these compounds had an effect on sperm motility experiments were performed using phorbol 12-myristate-13- acetate (PMA). The motility of epididymal spermatozoa following their incubation with varying concentrations of PMA are shown in Table 3.VI.

Table 3.VI. The effect of PMA on the motility of spermatozoa.

Concentration of PMA nM	Percent of Motile Spermatozoa			
	CAP(+)	CAP(-)	CAUD(+)	CAUD(-)
0	40.5 ± 2.5	2.5 ± 1.6	45.5 ± 2.5	27.0 ± 2.8
1	49.0 ± 1.5	3.5 ± 1.5	66.0 ± 1.8 ***	30.0 ± 1.8
10	58.0 ± 2.7 ***	4.0 ± 1.0	80.0 ± 3.5 ***	38.5 ± 2.7 *
100	78.0 ± 3.5 ***	4.0 ± 1.0	99.0 ± 2.6 ***	44.0 ± 3.0 **

Values are means ± SD (n=6).
CAP = Caput epididymal sperm + = + bicarbonate (15 mM)
CAUD = Caudal epididymal sperm - = no bicarbonate
* P<0.05
** P<0.01
*** P<0.001.

In the presence of bicarbonate PMA enhances the motility of spermatozoa isolated from the caudal epididymal region, at a level of 100 nM, by 117%, and this agent also significantly enhances the motility of caput epididymal spermatozoa when bicarbonate was present in the incubating medium. In the absence of exogenous bicarbonate, PMA stimulated the motility of caudal epididymal spermatozoa by 66% but had no significant effect on the motility of the immature caput epididymal spermatozoa. Thus it would appear that bicarbonate plays a role in the PMA-induced rise in sperm motility.

To investigate whether the PMA effect on motility depends upon the uptake of exogenous bicarbonate, the percent of motile caudal epididymal spermatozoa was studied in the presence of SITS (1 mM), an inhibitor of the bicarbonate/chloride exchanger and the results are shown in Table 3.VII.

Table 3.VII. The effect of SITS on PMA-stimulated motility.

Additions	Percent of Motile Spermatozoa
- (no bicarbonate)	28.0 ± 3.5
- (no bicarbonate) + PMA (100 nM)	43.0 ± 1.5 **
+ bicarbonate (15 mM) + PMA (100 nM)	99.0 ± 2.5 ***
+ bicarbonate (15 mM) + PMA (100 nM) + SITS (1 mM)	30.0 ± 4.0 +++

Values are means ± SD (n=6)
** P>0.01
***P>0.001 + bicarbonate and PMA v no bicarbonate + PMA
+++P>0.001 + bicarbonate, PMA and SITS v + bicarbonate and PMA

Again PMA enhanced the motility of caudal epididymal spermatozoa both in the absence and presence of external bicarbonate. However, this stimulation was abolished when these spermatozoa were incubated in the presence of SITS. Thus the entry of bicarbonate plays a key role in the PMA-induced stimulation of sperm motility.

For further evidence that protein kinase C plays a role in the stimulation of sperm motility an inhibitor of protein kinase C, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), (Rotem, *et al.*, 1990a) was tested for its effect on the motility of spermatozoa incubated in the presence of bicarbonate. In the presence of 20µg/cm^3 H-7 spermatozoa isolated from the caput section of the rat epididymis were totally immotile and the motility of caudal epididymal spermatozoa was inhibited by 177% from 87.0 ± 3.7 to 20.3 ± 4.1% (n=6).

CHAPTER 4

Factors Affecting Respiration and Motility of Spermatozoa from *Fucus serratus*.

4. Factors Affecting Respiration and Motility of Spermatozoa from *Fucus serratus*.

The aim of this study was to investigate the mechanisms involved in the activation of motility of spermatozoa from *Fucus serratus* upon their release into sea water. Before studies on the activation of the *Fucus serratus* spermatozoa could begin it was necessary to develop techniques to determine the number of spermatozoa in a sample and to measure their motility.

4.1. Measurement of Sperm Concentration.

Suspensions of spermatozoa from *Fucus serratus* can be quantified under a microscope, using a haemocytometer, but this is a tedious and lengthy procedure. In order to determine whether a spectrophotometric method was an accurate and efficient way of estimating the number of spermatozoa in a suspension the concentration of spermatozoa was determined both microscopically and by absorbance measurements at 340 nm. The number of spermatozoa in a suspension was adjusted to give $1x10^8$ spermatozoa/cm^3 and the absorbance of 10 separate aliquots of this suspension were read at 340 nm. It is clear from Figure 4-I that there is a positive correlation between these two methods of ascertaining the number of spermatozoa in a suspension (correlation coefficient of 0.84). The absorbance at 340 nm was 0.314 ± 0.022. Thus, this spectrophotometric method was subsequently used to measure sperm concentration.

4.2. Osmolarity of Sea water.

The experiments carried out in this study involved the measurement of O_2 uptake in ASW and in modified ASW. For this reason it was important that the osmolarity of the various sea water solutions was relatively constant.

Initially the osomolality of all the ASW solutions were checked and Table 4.I illustrates the figures which were obtained.

Table 4.I. The osmolality of sea water.

ASW		Osmolality (mOsm/litre)
Batch Number	1	1000
	2	1030
	3	1011
	4	980
	5	1015
	6	1000
	7	1043
	8	1000
	9	998
	10	976

It can be seen that these measurements were consitent. Subsequent batches of ASW and modified ASW were checked periodically and no samples were detected outside the accepted range.

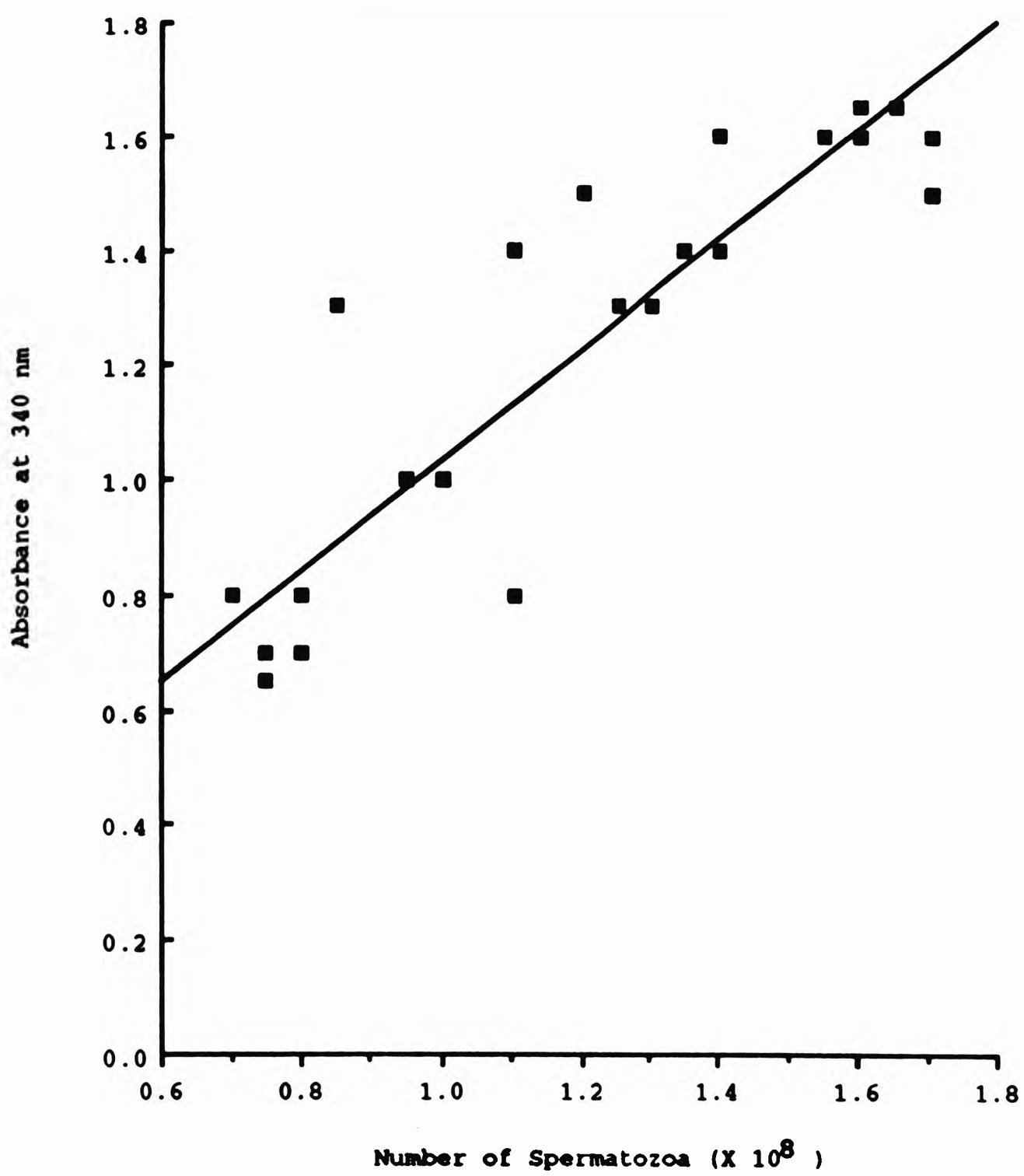
4.3. Respiration and Motility of Spermatozoa.

Spermatozoa are propelled forward by the beating of their flagella and ATP has been shown to provide the energy for this motion in spermatozoa from sea urchin, therefore motility and respiration of spermatozoa have the potential for being linked (Christen, *et al.*, 1982).

A possible relationship between respiration and motility in the spermatozoa of *Fucus serratus*, was investigated by using a Clarke oxygen electrode attached to a closed vessel to measure oxygen uptake and a microscope to assess motility. Figure 4-II illustrates that there is a positive correlation between O_2 uptake and the percentage of motile sperm (correlation coefficient of 0.81).

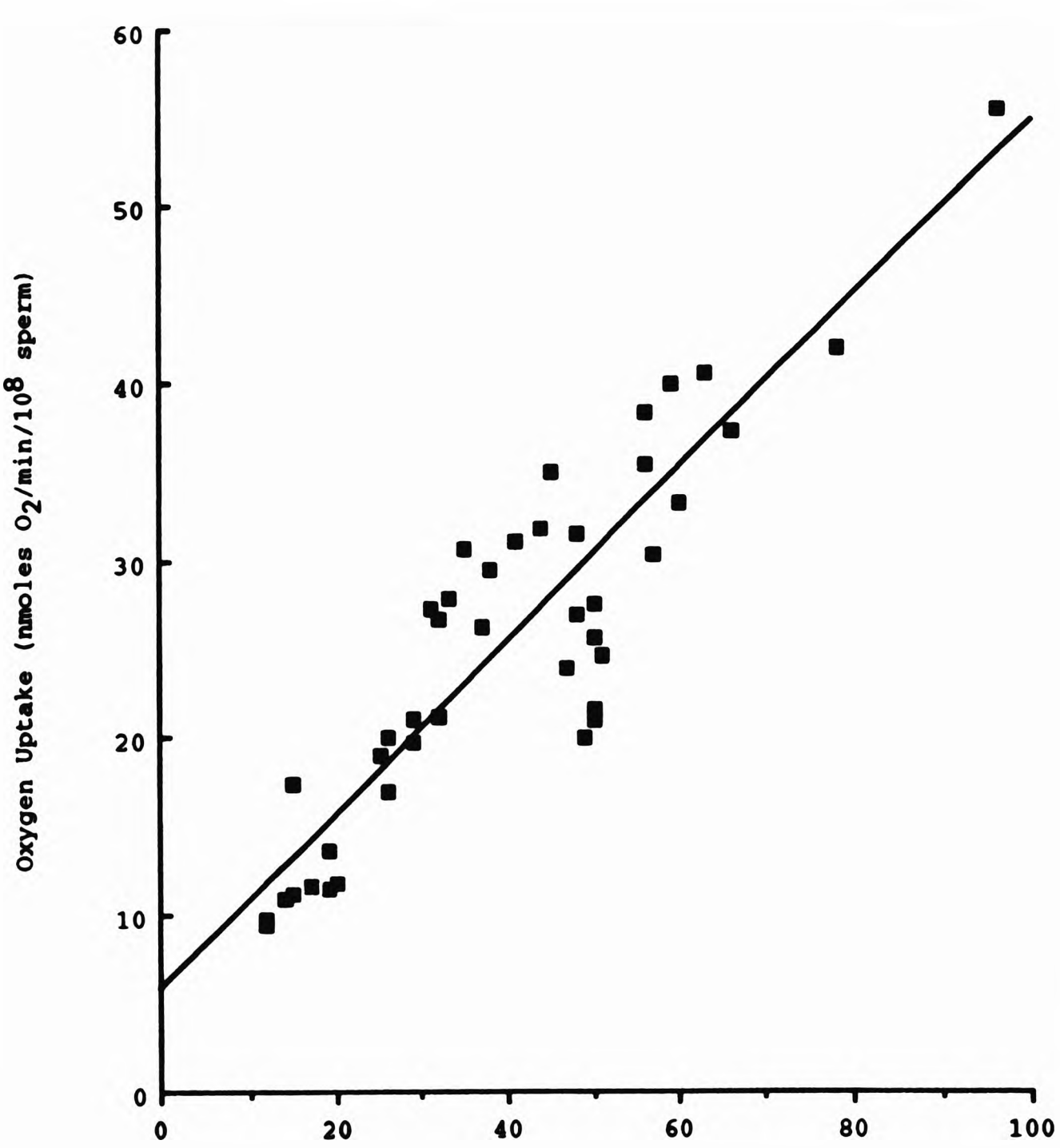
Thus it would appear that these two parameters are also linked in spermatozoa isolated from *Fucus serratus*.

Fig. 4.I. The Correlation between Two Methods of Estimating the Number of Spermatozoa in a Sample.



Washed spermatozoa were suspended in ASW and aliquots were removed and the number of spermatozoa in the suspension was estimated a. microscopically, using a haemocytometer and b. spectrophotometrically by reading absorbance at 340 nm, both procedures were carried out as described in Materials and Methods.

Fig. 4.II. The Correlation between the Oxygen Uptake and the Percent of Motile Spermatozoa in a Sample.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were suspended in ASW and aliquots were removed, motility was estimated microscopically and O_2 uptake was measured using an oxygen electrode. Both procedures were carried out as described in Materials and Methods.

4.4. The Effect of Temperature on Motility of Spermatozoa.

When suspensions of spermatozoa from *Fucus serratus* ($2x10^8$ spermatozoa/cm^3) were incubated in ASW at varying temperatures, motility was maximal at a temperature of 10°C (Table 4.II).

Table 4.II. The effect of temperature on the motility of spermatozoa from *Fucus serratus* incubated in ASW at pH 8.0.

Temperature of ASW °C	Percent of Motile Spermatozoa
4	8 ± 2
6	21 ± 2
10	85 ± 6
25	50 ± 5
37	23 ± 2
60	0

Values are means ± SD (n = 15)

4.5. The Effect of pH on Oxygen Uptake of Spermatozoa.

It has been reported that external pH affects motility and respiration of spermatozoa from sea urchin (Christen, *et al.*, 1983). The effects of pH on O_2 uptake and motility of spermatozoa from *Fucus serratus* at 10°C are shown in Table 4.III It is evident that both O_2 uptake and motility were maximal when the pH of the ASW was 8 and both were inhibited when the external pH was either more acidic or basic.

Table 4.III. The effect of external pH on the motility and oxygen uptake of spermatozoa from *Fucus serratus*.

pH	Percent of Motile spermatozoa	Oxygen Uptake nmoles O_2/min/10^8 sperm
5	18 ± 4	12.96 ± 1.84
6	30 ± 5	10.28 ± 1.82
7	51 ± 3	29.25 ± 4.15
8	85 ± 7	46.62 ± 3.53
9	54 ± 4.5	24.03 ± 2.41
10	40 ± 6	16.45 ± 3.25

Values are means ± SD (n = 15, from different batches of seaweed)

The initiation of respiration of sea urchin spermatozoa is dependent upon both the ionic composition and pH of the medium (Christen, ***et al.***, 1982) and as shown in Table 4.III the rate of respiration of spermatozoa from *Fucus serratus* is also dependent upon the pH of the ASW. Altering the external pH also alters the intracellular pH of sea urchin spermatozoa (Lee, *et al.*, 1982; Christen, *et al.*, 1982; 1983). Table 4.IV shows that decreasing the external pH of spermatozoa of *Fucus serratus* which causes acidification of the intracellular pH (Christen, *et al.*, 1982; 1983) decreases the rate of respiration both for coupled and FCCP-treated uncoupled spermatozoa.

Table 4.IV. The effect of external pH on coupled and uncoupled respiration.

Extracellular pH	Mean O_2 uptake nmoles O_2/min/10^8 spermatozoa	
	- FCCP	+ FCCP
5.0	4.23 ± 1.18	5.88 ± 1.76
6.0	14.98 ± 2.94	16.39 ± 1.76
7.0	32.90 ± 3.53	34.55 ± 3.29
8.0	42.01 ± 4.23	42.01 ± 4.44

Vales are means ± SD (n=6, from different batches of seaweed)

4.6. The Effect of the Ionic Composition of Sea water on Sperm Respiration and Motility.

Spermatozoa from *Fucus serratus* are released into sea water prior to fertilisation and therefore the ions present in sea water may play a role in the activation of motility and respiration of these spermatozoa. Suspensions of spermatozoa ($2x10^8$ spermatozoa/cm^3) were incubated, for 10-15 minutes, in ASW from which each ion is omitted in turn and the percent of motile sperm and O_2 uptake were measured (Table 4.V). In each experiment the ionic strength of the ASW remained the same, for example, N-methylglucamine replaced Na^+ in experiments requiring Na^+-free sea water and when the concentration of K^+ was varied, Na^+ was substituted for K^+, so that $[K^+] + [Na^+]$ was always 370 mM.

Table 4.V. The effect of the composition of artificial sea water on motility and oxygen uptake of spermatozoa from *Fucus serratus*.

Ion omitted	Percent of Motile Spermatozoa	Mean O_2 Uptake nmoles O_2/min/10^8 sperm
-	84 ± 4	46.82 ± 2.29
K^+	77 ± 5	43.29 ± 2.29
Ca^{2+}	25 ± 2 ***	15.60 ± 1.76 ***
Na^+	1 ± 0.25 ***	8.11 ± 2.26 ***
Mg^{2+}	29 ± 2 ***	17.77 ± 1.59 ***

Values are means ± SD (n = 10).
*** P< 0.001.

These results show that when spermatozoa were incubated in sodium free ASW percentage motility and respiration were decreased by 100% and 83% respectively. External Ca^{2+} was also important for the initiation of motility and respiration in these spermatozoa and when this ion was omitted from ASW the percentage of motile sperm was decreased by 70% and oxygen uptake by 67%. Mg^{2+} also appears to play a role in the activation of spermatozoa as in its absence sperm motility was inhibited by 65% and O_2 uptake by 62%.

These results suggest that the presence of external Na^+ is essential for spermatozoa to acquire motility and this is linked to the rate of respiration. The presence of Ca^{2+} and Mg^{2+}, although enhancing motility and respiration, are not essential.

The effect of varying concentrations of Na^+, in ASW, on oxygen uptake in the spermatozoa of *Fucus serratus* is shown in Figure 4.III. Spermatozoa suspended in Na^+-free ASW for 10 - 15 minutes had an O_2 uptake of 8.10 ± 0.59 nmoles O_2/min/10^8 spermatozoa and were immotile, as measured microscopically. Increasing

the Na^+ concentration in ASW resulted in an elevation in O_2 consumption which reached an optimum at 360 mM Na^+ which is the Na^+ concentration in natural sea water. Further increases in Na^+ concentration resulted in a fall in oxygen consumption. The percentage of motile sperm also increased from 0 to 85% as the amount of Na^+ present in ASW rose from 0 to 360 mM, after which motility fell to 65% when 500 mM Na+ was present in the sea water.

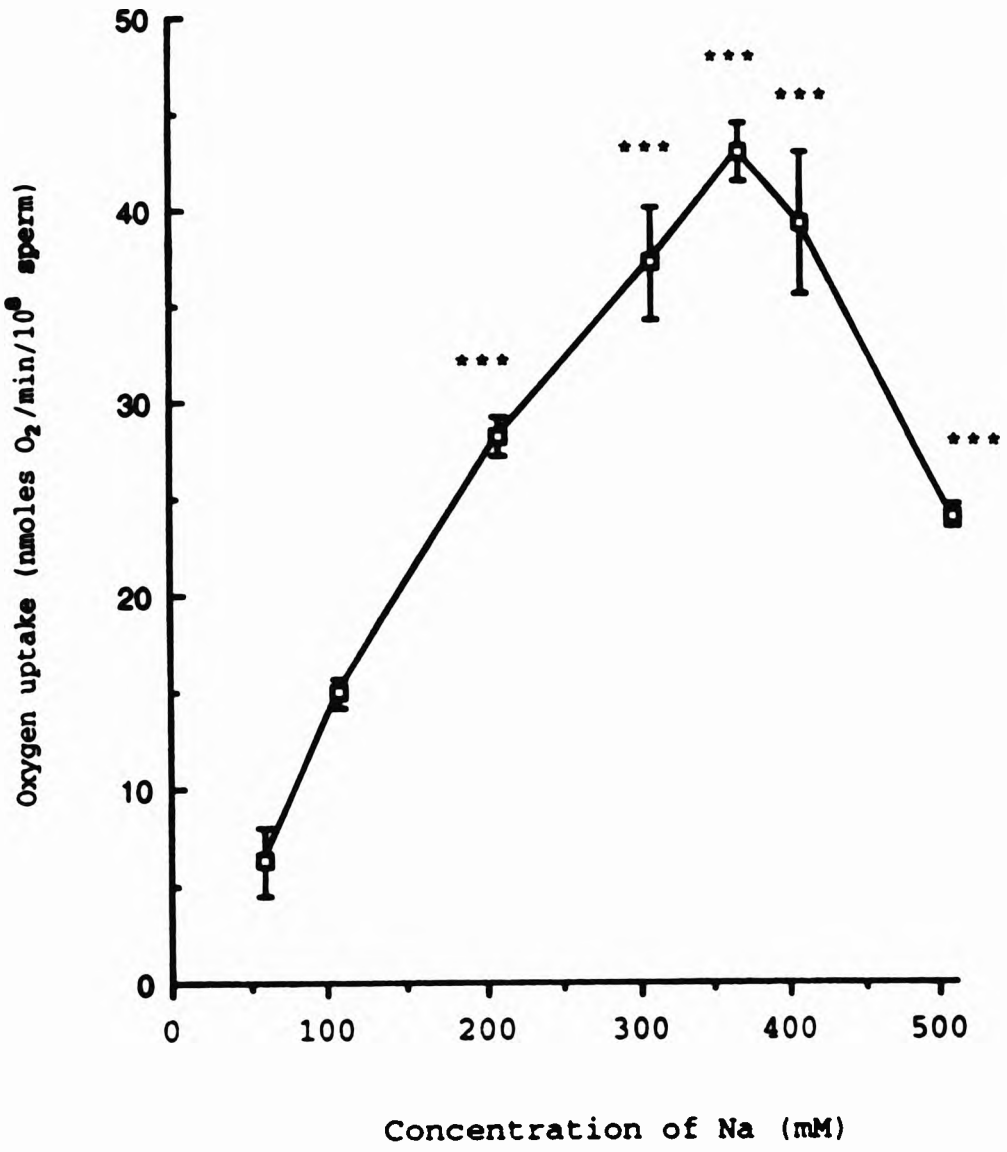
As previously stated the O_2 uptake of spermatozoa is lower in Na^+-free sea water (ONaASW) than in its presence. However, when Na^+ was re-introduced to a suspension of spermatozoa in ONaASW O_2 uptake appeared to rise within the first two minutes after Na^+ had been re-introduced (Table 4.VI). However, such an addition would increase the osomolarity which in turn would cause a significant decrease in O_2 solubility. This means that the value of 282.01 µmoles/litre could be artificially high. However it is unlikely that this would totally account for the difference recorded between the O_2 uptake in the presence and absence of Na^+.

Table 4.VI. The effect of Na^+ on O_2 uptake of spermatozoa.

Composition of ASW	Mean O_2 uptake nmoles O_2/min/10^8 spermatozoa
Control	43.59 ± 1.65
ONaASW	8.11 ± 2.26 ***
+ 360 mM Na^+	13.25 ± 3.03

Values are means ± SD (n = 10).
*** P<0.001.

Fig. 4.III. The Effect of the Na Concentration of ASW on the Oxygen Uptake of Spermatozoa.



Washed spermatozoa (1-2 x10^8 spermatozoa/cm^3) were incubated in ONaASW at 10 °C for 10 min in the presence of increasing concentrations of Na^+ added from a 10x stock solution of NaCl. O_2 uptake was measured as under Materials and Methods. Values are means ± SD (n=10).
*** P< 0.001.

4.7. The Effect of n-hexane on Sperm Motility and Respiration.

The interaction between *Fucus* gametes is mediated by the secretion of a pheromone-like sperm chemo-attractant by the eggs (Müller and Seferiadis, 1977). A variety of simple hydrocarbons including n-hexane, ethers and esters mimic the natural chemo-attractant (Cook, *et al.*, 1951) although the sensitivity of *Fucus* sperm to n-hexane is some three orders of magnitude less than the natural chemo-attractant, Fucoserraten.

The effect on O_2 uptake and motility of spermatozoa of *Fucus serratus* was ascertained in the presence of ASW containing 1 mM or 10 mM n-hexane.

Table 4.VII. The effect of n-hexane on the O_2 uptake and motility of spermatozoa.

Conditions	Percent of Motile Spermatozoa	Mean O_2 uptake nmoles O_2/min/10^8 spermatozoa
Control	85 ± 1.50	45.24 ± 2.41
+ 1 mM n-hexane	99 ± 0.35	137.24 ± 2.22 ***
+ 10 mM n-hexane	94 ± 0.50	85.94 ± 1.59 ***

Values are means ± SD (n = 10).
*** P<0.001.

The results in Table 4.VII show that both O_2 uptake and sperm motility were stimulated when incubated with n-hexane by 203% and 11% respectively in the presence of 10 mM n-hexane and by 90% and 16% respectively by 1 mM n-hexane. In both cases this hydrocarbon enhanced O_2 uptake to a much greater extent than it stimulated the percent of motile sperm and, therefore, an increase in the rate of respiration may be required first before there is an increase in motility.

CHAPTER 5

The Involvement of Ion Channels in Activation of Motility of Spermatozoa from *Fucus serratus*.

5. The Involvement of Ion Channels in Activation of Motility of Spermatozoa from *Fucus serratus.*

It is clear from previous results (section 4.4 and 4.5) that the pH and ionic composition of the surrounding medium is important in initiating sperm motility. This would suggest that ion channels play a crucial role in this activation process.

5.1. The Na^+/H^+ Exchanger.

From the results already discussed it would appear that Na^+ is a major trigger of sperm motility. External Na^+ crosses membranes via a Na^+/H^+ exchanger, which serves as a regulator of pHi in most cells and the rate of Na^+/H^+ exchange is strongly dependent on the internal H^+ concentration. Aronson and colleagues (1982; 1985) have proposed that cytoplasmic H^+ acts as an allosteric activator of the Na^+/H^+ exchanger.

The diuretic drug amiloride is an inhibitor of the Na^+/H^+ exchange activity and acts by competing with Na^+ for the external binding site on the exchanger. When amiloride was added to a suspension of spermatozoa from *Fucus serratus* (2x108 spermatozoa/cm^3) motility decreased as shown in Table 5.I.

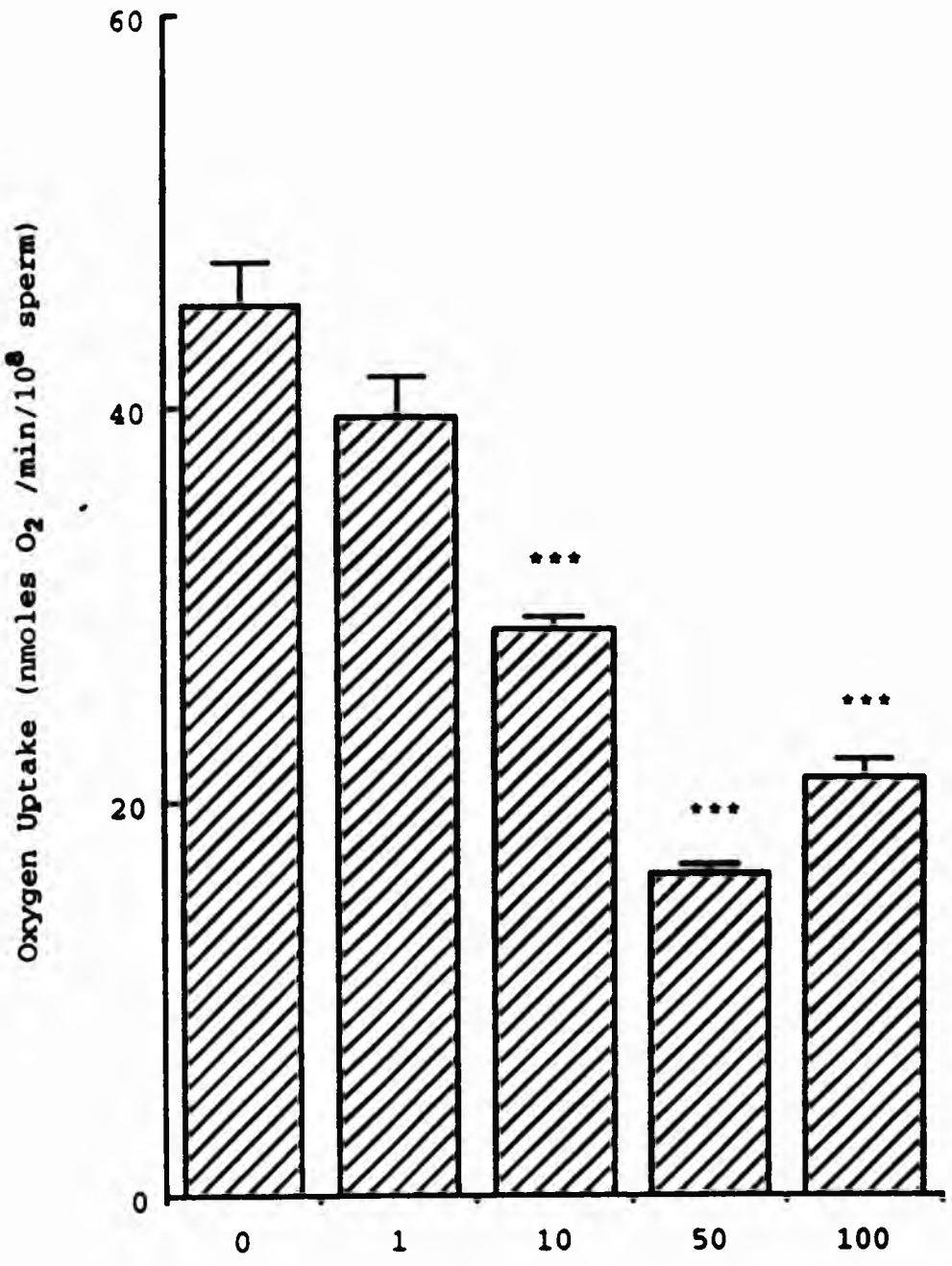
Table 5.I. The effect of amiloride on the motility of spermatozoa from *Fucus serratus*.

Concentration of amiloride (μM)	Percent of Motile Spermatozoa
0	83 ± 5.5
1	70 ± 4 ***
10	49 ± 5 ***
50	27 ± 3.5 ***
100	34 ± 2 ***

Values are means ± SD (n=15, from different batches of seaweed).
*** $P<0.001$.

Amiloride inhibits the number of motile sperm by 16% even when present at the very low concentration of 10^{-6}M. However, maximum inhibition (67%) was reached when 50 μM amiloride was included in the incubation medium. Amiloride also caused a decrease in O_2 uptake by these spermatozoa. Results in Figure 5.I show that spermatozoa which had been incubated in ASW in the absence of amiloride respired at a rate of 45.18 ± 2.17 nmoles O_2/min/10^8 spermatozoa, whereas the addition of amiloride (final concentration 50 μM) produced a significant ($P<0.001$) decrease in O_2 uptake of 64% to 16.33 ± 0.59 nmoles O_2/min/10^8 spermatozoa. Thus the same concentration of amiloride (50 μM) produced the maximum inhibition of both respiration and sperm motility.

Fig. 5.I. The Effect of Amiloride on the Oxygen Uptake of Spermatozoa.

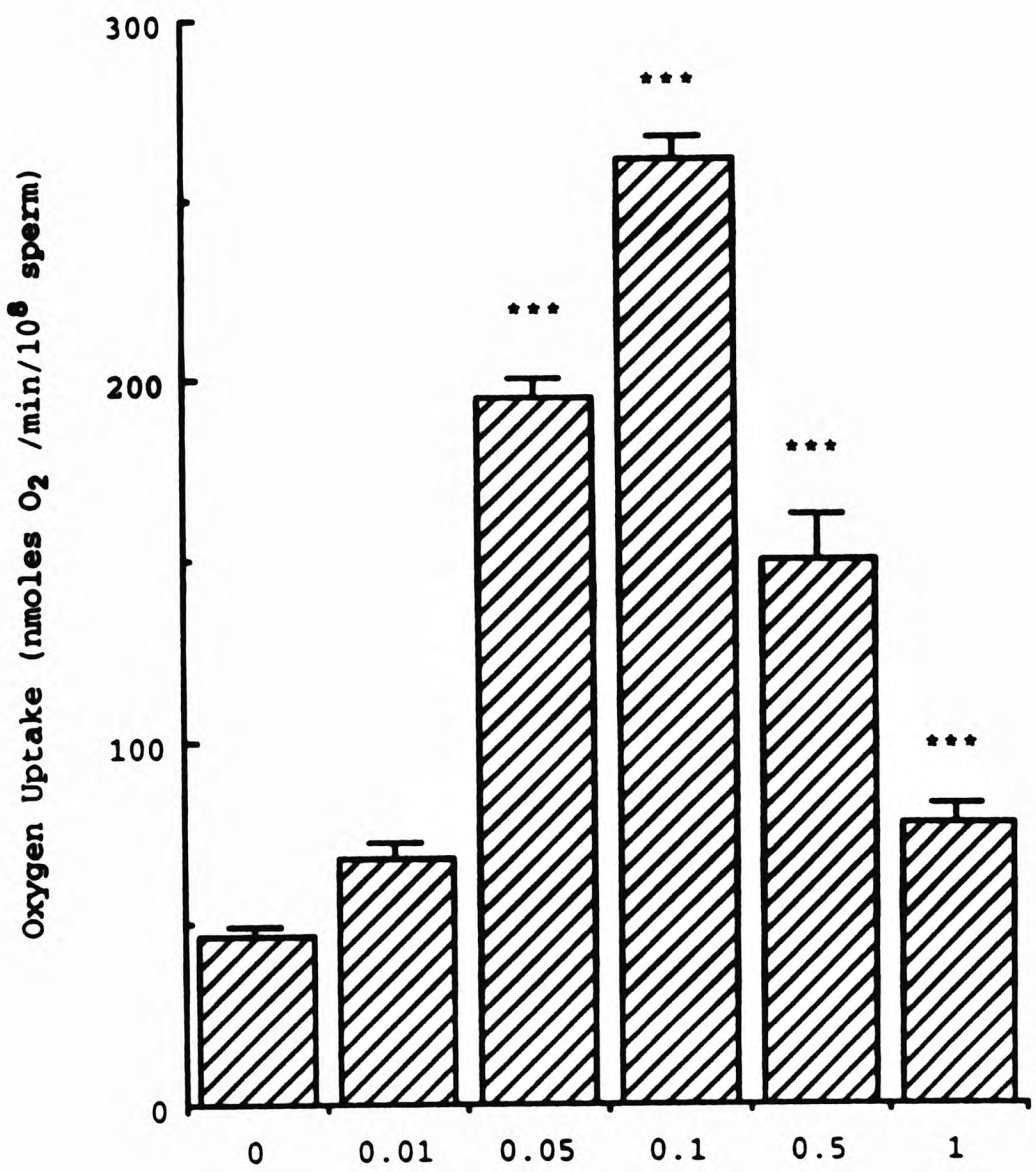


Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated at 10°C for 15 min in the presence of varying concentrations of amiloride, additions were made from 100x stock solutions. Values are means ± SD (n=10, from 10 different batches of seaweed).
*** $P < 0.001$.

The monovalent cationic ionophore monensin, which catalyses electrically neutral Na^+/H^+ exchange across cell membranes (Pressman, 1976; Hansbrough and Garbers, 1981), activates the O_2 uptake of spermatozoa from *Fucus serratus*. The results in Figure 5.II show that the O_2 uptake in the absence of monensin was 46.82 ± 2.29 nmoles O_2/min/10^8 spermatozoa whereas 0.1 µM monensin significantly ($P<0.001$) increased O_2 uptake by 457% to 261.03 ± 5.78 nmoles O_2/min/10^8 spermatozoa.

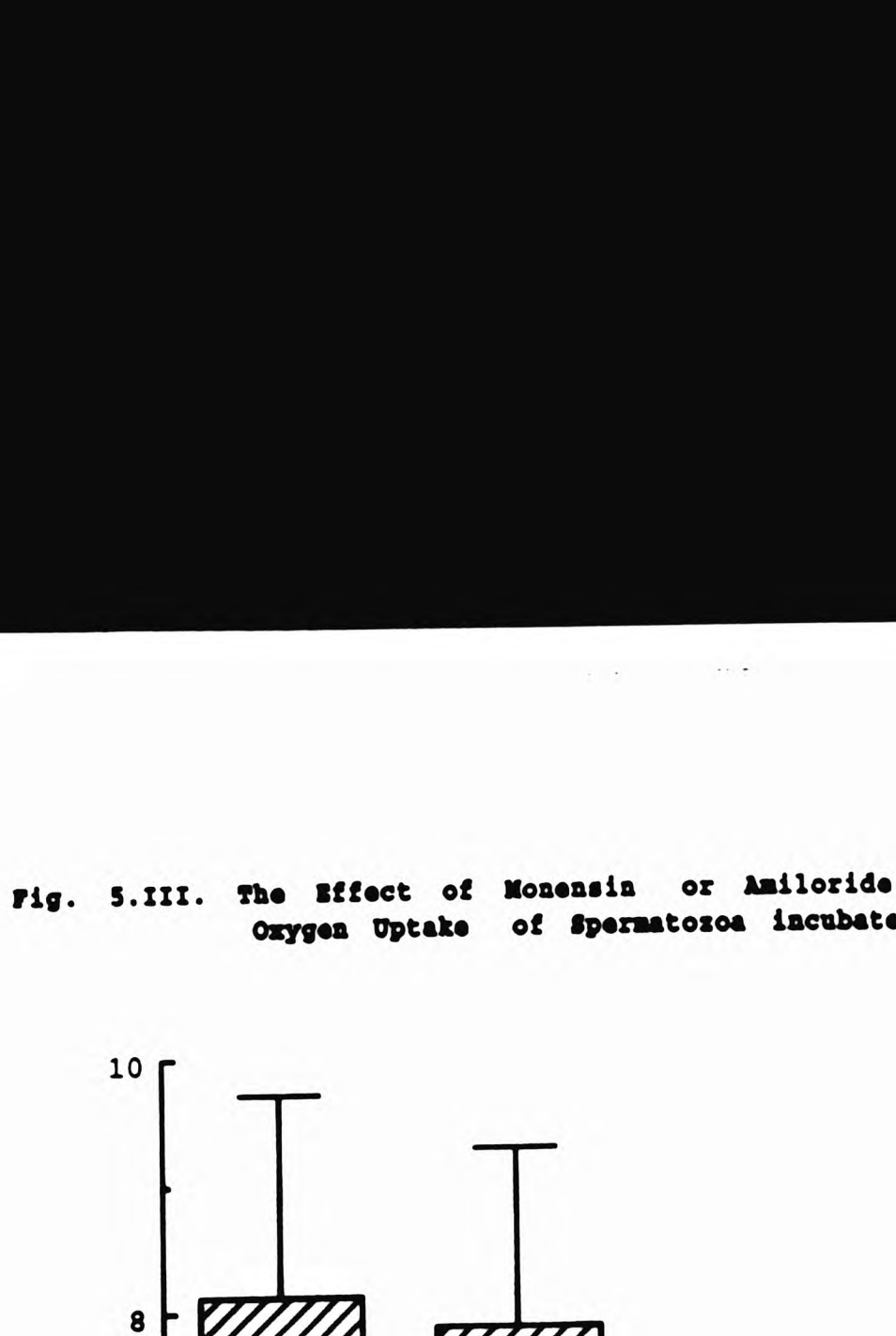
In sea urchin sperm a Na^+/H^+ exchanger participates in the regulation of intracellular pH, which is dependent on the presence of external Na^+ (Darszon, *et al.*, 1987; García-Soto, *et al.*, 1987). The investigations already described with amiloride and monensin were carried out in ASW, containing the complete complement of salts. It seemed appropriate to test these two agents in Na^+-free ASW where uptake of O_2 by spermatozoa is normally absent or very low. The addition of amiloride (final concentration: 50 µM) to spermatozoa incubated in ONaASW resulted in a further decrease in O_2 uptake by 78%, whereas the addition of monensin (final concentration: 0.1 µM) to spermatozoa in ONaASW had no significant effect on O_2 uptake (Figure 5.III).

Fig. 5.II. The Effect of Monensin on the Oxygen Uptake of Spermatozoa.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated at 10°C for 15 min in the presence of varying concentrations of monensin, additions were made from 100x stock solutions. Values are means ± SD (n=10, from 10 different batches of seaweed).

Fig. 5.III. The Effect of Monensin or Amiloride on the Oxygen Uptake of Spermatozoa incubated in ONaASW.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated in ONaASW at 10°C for 15 minutes in the presence of either amiloride (50 µM) or monensin (0.1 µM). Values are means ± SD (n=10, from 10 different batches of seaweed).

5.2. The Na^+/K^+ ATPase pump.

The Na^+/H^+ exchange is driven by the transmembrane Na^+ gradient which in turn is generated by Na^+/K^+ ATPase.

In many systems, during the Na^+/ H^+ exchange one H^+ is extruded for every Na^+ entering the cell and this Na^+ is then pumped out again by the ATP-dependent Na^+/ K^+ ATPase. If this Na^+/K^+ pump is present and responsible for the efflux of Na^+ in spermatozoa from *Fucus serratus* then motility and O_2 uptake should be slowed down under one or more of the following conditions:

I. in the presence of an inhibitor of this pump,

II. by substituting Li^+ for Na^+,

III. after depletion of cellular ATP.

I. Ouabain, a compound isolated from certain plants, is a potent inhibitor of the Na^+/K^+ pump. It specifically binds to the outer surface of the Na^+/K^+ ATPase and blocks both ion translocation and ATP hydrolysis. The effect of ouabain on both the motility and O_2 uptake of spermatozoa from *Fucus serratus* was measured and the results are shown in Table 5.II.

Table 5.II. The effect of ouabain on the motility and O_2 uptake of spermatozoa of *Fucus serratus*.

Concentration of Ouabain (mM)	Percent of Motile Spermatozoa	Mean O_2 Uptake nmoles O_2/min/10^8 sperm
0	80 ± 9	54.66 ± 2.55
0.01	56 ± 8 *	35.09 ± 4.48 ***
0.1	22 ± 3 ***	21.34 ± 1.70 ***
1	32 ± 6 ***	27.46 ± 2.51 ***
10	15 ± 2 ***	8.21 ± 1.43 ***

Values are means ± SD (n = 10).
* P<0.01
*** P<0.001.

The results shown in Table 5.II indicate that ouabain effectively inhibits sperm motility even when present at low concentrations (10^{-5}M), but the greatest inhibition of 81% is observed at the highest concentration used of 10 mM.

The inclusion of ouabain in the incubation media also resulted in a decrease in O_2 uptake. From the results in Table 5.II it can be seen that in ASW O_2 uptake of spermatozoa was subject to 85% inhibition by 10 mM ouabain. Even in the presence of low concentrations of ouabain (0.01 mM) 36% inhibition in O_2 consumption occurred. It is clear that the inhibition of O_2 uptake by ouabain takes effect immediately, as suspensions of spermatozoa initially allowed to respire for 2 minutes exhibited an immediate fall in O_2 uptake (31%) on addition of ouabain (10 mM). Additionally, ouabain inhibits by 79% the very low O_2 uptake exhibited by spermatozoa suspended in Na+-free ASW (Table 5.III.). Thus ouabain is a potent inhibitor of O_2 uptake in spermatozoa from *Fucus serratus*.

Table 5.III. The effect of ouabain on O_2 uptake of spermatozoa incubated in ONaASW.

Composition of ASW	Mean O_2 uptake nmoles O_2/min/10^8 spermatozoa
ONaASW	8.11 ± 2.29
+ 10 mM ouabain	1.59 ± 0.59 ***

Values are means ± SD (n = 10, from different batches of seaweed).
*** P<0.001.

II. Lithium is a very good substitute for Na^+ in Na^+/H^+ counter movements (Ives *et al* 1983; Paris and Pouyssegur, 1983), but intracellular Li^+ is only pumped out of cells at a very limited rate by the Na^+/K^+ ATPase pump (Dunham and Sengh, 1977). Previous studies on various cell types have shown that Na^+ enters the cell in exchange for H^+ ions which leads to the internal alkalisation of the cell (Gatti and Christen, 1985) and this intracellular alkalisation would probably be reduced in the presence of lithium.

Table 5.IV. The motility and O_2 uptake of spermatozoa from *Fucus serratus* in the presence of lithium ions.

	Percent of Motile Spermatozoa	Mean O_2 uptake nmoles O_2/min/10^8 spermatozoa
Control	89 ± 3	47.18 ± 1.94
ONaASW	1 ± 0.25 ***	8.11 ± 2.26 ***
Li-ONaASW	22 ± 4 ***	22.38 ± 3.53 ***

Values are means ± SD (n = 10, different batches of seaweed).
*** $P<0.001$.

The results in Table 5.IV show that both O_2 uptake and percent of motile sperm were inhibited by 53% and 75% respectively, when Na^+ was replaced by Li^+ in ASW. However, the spermatozoa were more active in the presence of ONaASW containing Li^+ than spermatozoa suspended in ONaASW alone implying that Li^+ can partially substitute for Na^+ in spermatozoa of *Fucus serratus*, this is presumably because the initial countermovements would cause the expulsion of H^+, however as Li^+ cannot be cycled it cannot maintain this expulsion. Therefore, it would appear that the Na^+/K^+ ATPase pump may be only one of the components involved in activating spermatozoa of *Fucus serratus*.

5.3. The Bicarbonate/Chloride Exchanger.

There are two exchangers which involve bicarbonate, the first is an acid extruder which acts by exchanging external Na^+ and HCO_3^- for internal H^+ and Cl^-, the second is an alkali extruder which exchanges intracellular HCO_3^- for extracellular Cl^-. The Na^+, HCO_3^--Cl^-, H^+ exchanger is inhibited by stilbene derivatives such as 4-acetamido-4`-isothiocyanostilbene-2,2`-disulfonic acid (SITS).

The results in Table 5.V show that spermatozoa incubated in ASW in the presence of 0.01 mM SITS had an O_2 uptake of 16.85 ± 1.20 nmoles O_2/min/10^8 spermatozoa which represented an 60% inhibition of respiratory activity. Higher concentrations of SITS (0.1 - 1 mM) did not markedly increase this inhibition. 40% of the inhibitory effect of SITS occurs within the first minute after its addition to spermatozoa. SITS also inhibits motility in these sperm as shown in Table 5.V. Maximal inhibition of motility occurred when 0.1 mM SITS was present in the incubating ASW, where percent of motile sperm fell from 85 ± 2% to 23 ± 1% , a 73% inhibition of sperm motility.

Table 5.V. The effect of SITS on the motility and O_2 uptake of spermatozoa.

Concentration of SITS (mM)	Percent of Motile Spermatozoa	Mean O_2 Uptake nmoles O_2/min/10^8 sperm
0	85 ± 2	38.25 ± 2.30
0.01	30 ± 1 ***	16.85 ± 1.20 ***
0.1	23 ± 1 ***	11.76 ± 0.60 ***
0.5	38 ± 2 ***	14.52 ± 1.27 ***
1	47 ± 2 ***	18.67 ± 1.76 ***

Values are means ± SD (n = 15). *** P<0.001.

5.4. Ca^{2+} Channels.

One of the ways by which external Ca^{2+} enters a cell is through voltage-dependent Ca^{2+} channels. A known inhibitor of these channels is verapamil. Suspensions of spermatozoa incubated in ASW containing varying concentrations of verapamil showed a decrease in both O_2 uptake and the percent of motile spermatozoa. The results are shown in Table 5.VI.

Table 5.VI. The effect of Verapamil on the motility and O_2 uptake of spermatozoa.

Concentration of Verapamil (μM)	Percent of Motile Spermatozoa	Mean O_2 Uptake nmoles O_2/min/10^8 sperm
0	89 ± 3.2	46.35 ± 3.14
0.1	43 ± 1.4 ***	20.61 ± 4.91 ***
1	39 ± 0.9 ***	9.68 ± 1.92 ***
10	36 ± 2.0 ***	14.37 ± 4.42 ***
100	28 ± 4.1 ***	12.97 ± 4.69 ***
500	23 ± 2.3 ***	10.69 ± 1.94 ***
1000	14 ± 2.0 ***	7.21 ± 1.47 ***

Values are means ± SD (n = 15, from different batches of seaweed).
*** P<0.001.

The inhibition of O_2 uptake and percentage motility increased from 44% and 57% respectively at 0.1 μM verapamil to 84% for both parameters at a concentration of 1 mM.

CHAPTER 6

Intracellular Mechanisms Associated with Activation of Motility.

6. Intracellular Mechanisms Associated with Activation of Motility.

6.1. Na^+-dependent H^+release.

It has been demonstrated in spermatozoa from sea urchin that the extrusion of H^+ produces an increase in cytoplasmic pH (Lee, 1984 a & b) and this is a prerequisite for activation of sperm motility. It is clear from these results and those of others that this process is Na^+ dependent and relies upon the activation of the Na^+/H^+ exchanger. For example the activation of sperm motility accompanied by acid extrusion which occurs when these sperm are released into sea water does not occur in the absence of external Na^+ (Nishioka and Cross, 1978).

6.1.1. Characteristics of Na^+-dependent H^+release in Intact spermatozoa.

Figure 6.I shows results of experiments in which the pH of *Fucus serratus* sperm suspensions, in Na^+-free ASW, were monitored using a pH electrode. Samples of spermatozoa were also removed at various time intervals for quantitative estimation of sperm motility. As previously illustrated spermatozoa suspended in Na^+-free ASW are virtually immotile. The addition of 360 mM NaCl to a suspension of spermatozoa in 0NaASW resulted in a decrease in the pH of the medium in which the sperm was suspended, by 0.4 of a pH unit, from 8 to 7.6 suggesting the release of acid from the spermatozoa. The percent of motile sperm increased to over 80%. The release of acid could be divided into two phases; an initial rapid release for 30 seconds was followed by a slower rate of release (Figure. 6.Ia,a_1 and a_2 respectively). The latter phase involving the slow release of H^+ was inhibited in the presence of potassium cyanide (KCN), (Figure. 6.Ib) suggesting that this slow release was associated with respiration.

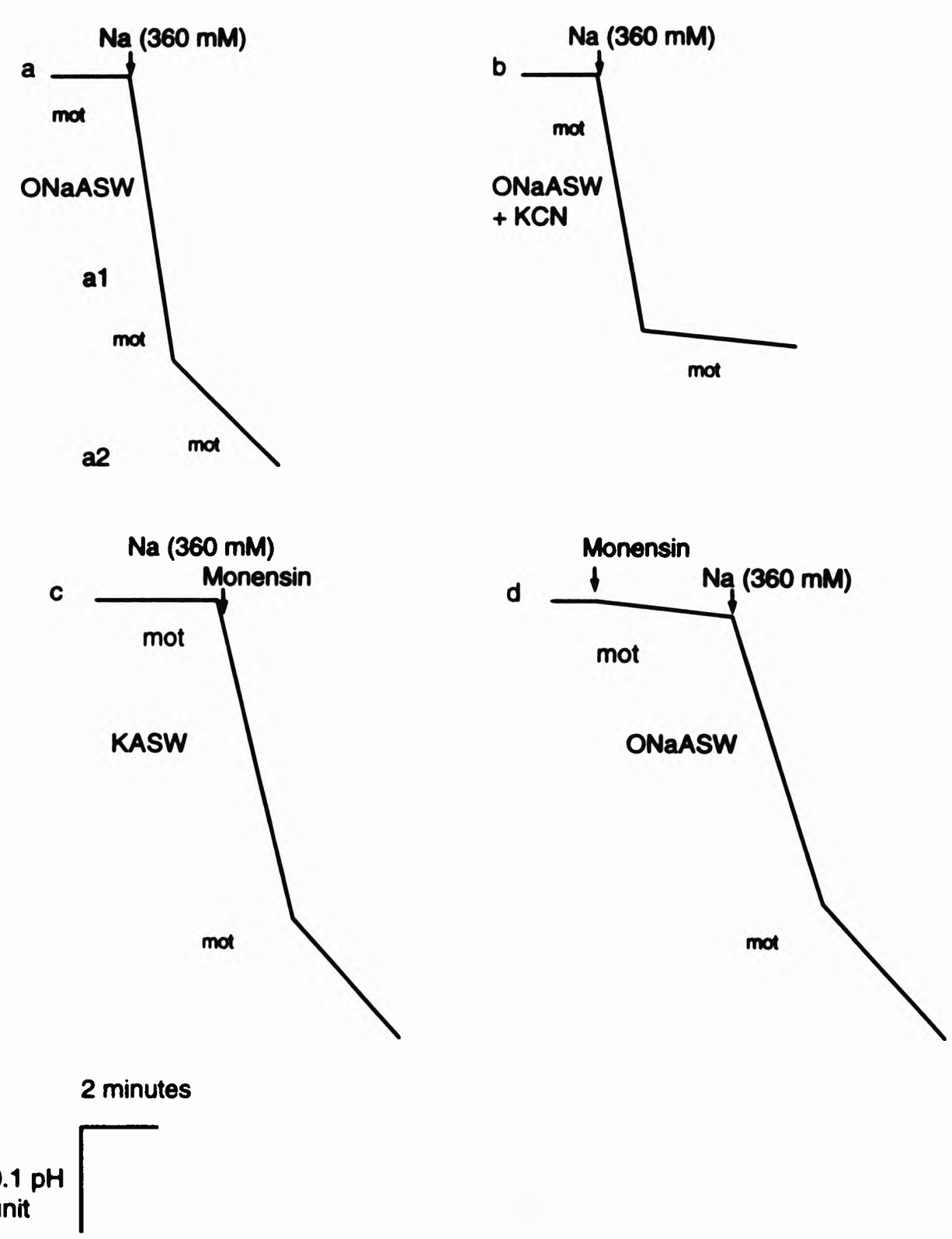
The efflux of H^+ was also sensitive to the K^+ concentration in the ASW. When Na^+ (360 mM) was added to spermatozoa suspended in Na^+-free ASW containing 160 mM K^+ (normal concentration of K^+ in sea water is 10 mM) there was neither an efflux of acid nor an increase in percentage motility, in fact motility was inhibited by 60% compared to the motility of spermatozoa incubated in normal ASW (Figure. 6.Ic). On the other hand, in the presence of a high K^+ concentration, the concentration of Na^+ remained normal (360 mM), acid release was induced by the addition of the Na^+/H^+ exchange ionophore, monensin (50 µM) (Figure. 6.Ic) and this was accompanied by an increase in motility to approximately 98%. There was no monensin induced H^+ release in the complete absence of Na^+ (Figure 6.Id), but subsequent addition of 360 mM NaCl to the sperm suspension resulted in a decrease in the pH 0.3 of a pH unit and an increase in sperm motility to 95%.

6.1.2. Characteristics of Na^+-dependent H^+ Release in Isolated Flagella.

Spermatozoa are propelled forward by their flagella and if acid extrusion plays a role in motility then it is very probable H^+ would be released from isolated flagella. The characteristics of the efflux of H^+ from isolated flagella are shown in Figure 6.II and are similar to those of intact spermatozoa. There was no decrease in the pH of a suspension of isolated flagella suspended in Na^+-free ASW (Figure 6.IIa). However, on addition of 360 mM NaCl the pH decreased by 0.35 of a pH unit. As with intact spermatozoa no acid was released by the flagella when placed in ASW containing a high concentration of KCl (final concentration 160 mM) (Figure 6.IIb). The pH of the flagella suspension remained unchanged in the presence of monensin (50 µM) when incubated in 0NaASW, but upon the introduction of Na^+ to a suspension of isolated flagella the pH decreased by 0.3 pH units (Figure. 6.IIc). Thus it seems probable that the acid is released from the flagella but release from other regions cannot be ruled

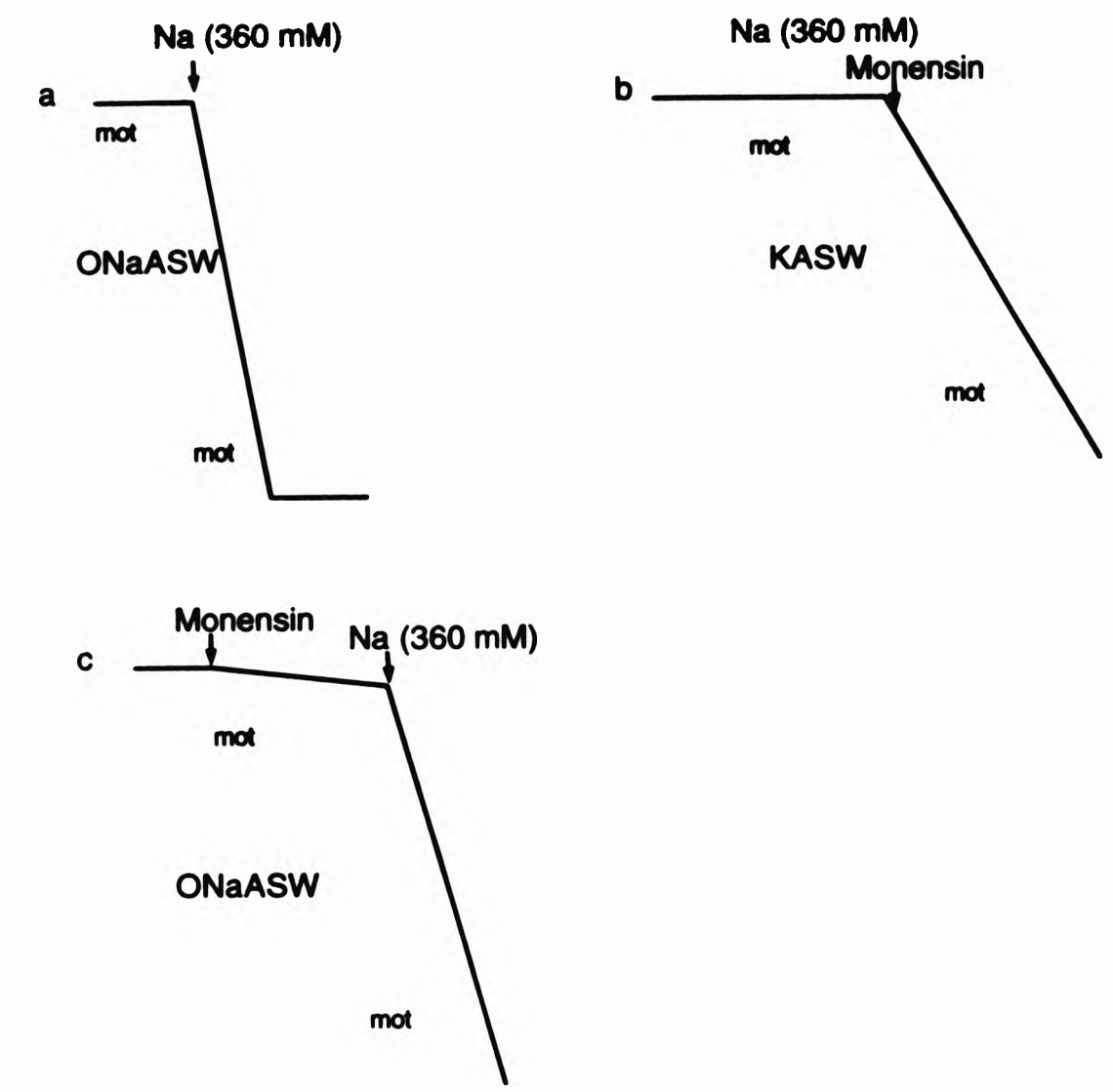
out. However, contamination of the flagella suspension by sperm bodies or intact spermatozoa was low, as monitored microscopically.

Figure 6.I. Characteristics of acid extrusion from intact spermatozoa.



A 250µl aliquot of sperm suspension was added to 5 cm^3 of Na+-free ASW (ONaASW) or high K^+ (160 mM) ASW (KASW) and the pH monitored. At various time intervals aliquots were removed for motility assay (mot). When indicated sodium (Na) or monensin were added in small aliquots from concentrated stock solutions (pH 8) to give final concentrations of 360 mM NaCl and 50 µM monensin respectively. KCN (final concentration: 1 mM), when tested, was added to the ONaASW before the addition of spermatozoa.

Figure 6.II. Characteristics of acid extrusion from isolated flagella.



A 250 µl aliquot of isolated flagella was suspended in 5 cm^3 of ONaASW or high K^+ (160 mM) ASW (KASW) and the pH monitored. At various time intervals aliquots were removed for motility assay (mot). When indicated sodium (Na) or monensin were added in small quantities from concentrated stock solutions (pH 8) to give final concentrations of 360 mM Na+ and 50 µM monensin respectively.

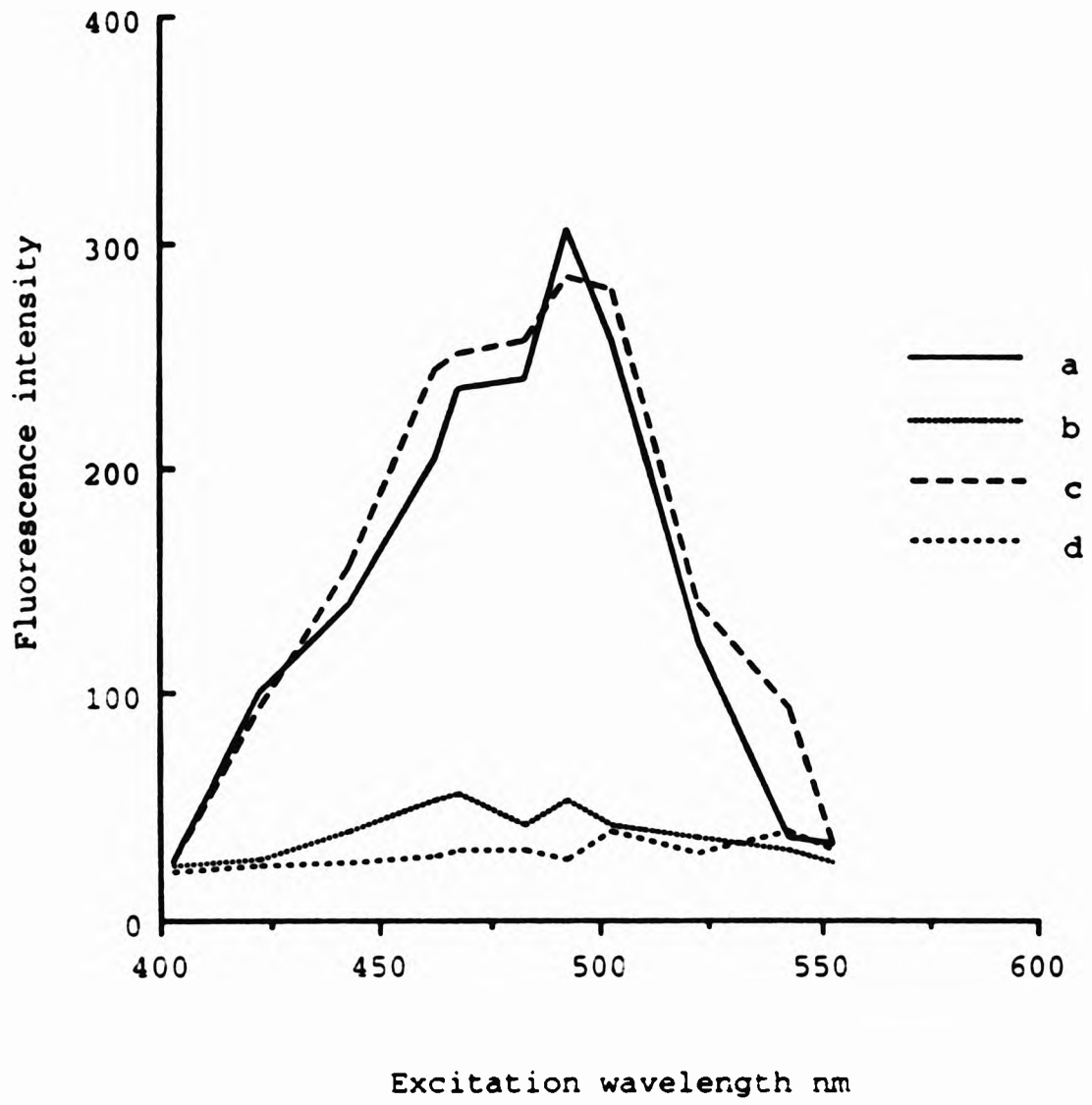
6.2. Estimation of Intracellular pH.

6.2.1. Uptake and Hydrolysis of BCECF-AM by Spermatozoa from *Fucus serratus*.

The decrease observed in the pH of the medium when the motility of spermatozoa from *Fucus serratus* increased may well reflect an increase in internal pH. In the present studies the fluorescent probe BCECF-AM was used to monitor changes in internal pH of sperm from *Fucus serratus*. BCECF is a pH-sensitive fluorescent probe which is taken up by spermatozoa in its acetoxymethylester form and hydrolysed to BCECF once inside the cell, which ensures it is trapped within the cell.

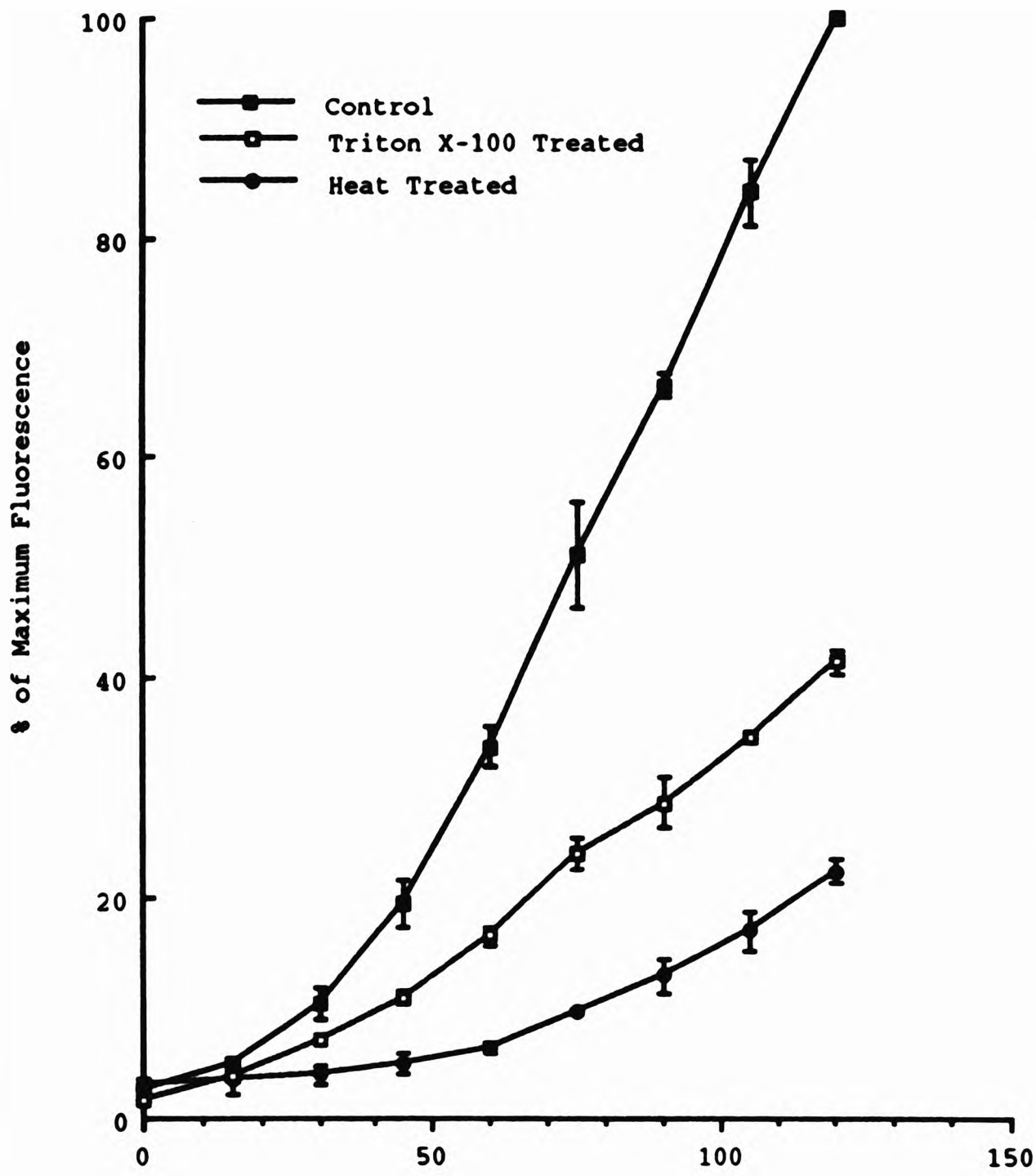
Suspensions of spermatozoa from *Fucus serratus* were incubated with BCECF-AM (5 μM) for 3 hours and then extensively washed to free them of external probe. The fluorescence intensity of spermatozoa loaded with BCECF-AM was then measured, over the excitation range 300 → 600 nm, with emission set at 550 nm. The results in Figure 6.III show that the excitation peak of these loaded spermatozoa was 490 nm, which is characteristic of the free acid hydrolysis product BCECF whereas, the excitation peak of the unhydrolysed acetylmethyl ester form of BCECF is 465 nm (Fig. 6.III). Therefore, after 3 hours *Fucus serratus* spermatozoa have taken up BCECF-AM and it has become hydrolysed to BCECF. Further evidence that BCECF-AM is hydrolysed intracellularly to BCECF was obtained from experiments in which the increase in fluorescence intensity was monitored over a period of time (Figure 6.IV). The increase normally observed was inhibited by lysing the sperm during incubation with the detergent Triton X-100 (0.1% v/v) or following inactivation of the sperm by prior heat treatment (Figure 6.IV). The probe did not affect O_2 uptake by the spermatozoa when compared with control sperm over a period of 5 hours (Figure 6.V), indicating that there was little or no adverse effect of the BCECF-AM hydrolysis products on cellular function.

Figure 6.III. Excitation Spectra of spermatozoa incubated with BCECF-AM.



Excitation spectra (emission was set at 550 nm) for:
a = BCECF- free acid (5 µM);
b = BCECF-AM (5 µM);
c = Spermatozoa from *Fucus serratus* labelled with BCECF-AM (5 µM);
d = Autofluorescence of spermatozoa from *Fucus serratus*.
All the above scans were carried out in ASW. Spermatozoa were incubated with BCECF-AM (5 µM) for 3 hours at 10°C, washed three times in ASW to remove extracellular dye and resuspended in ASW before scanning.

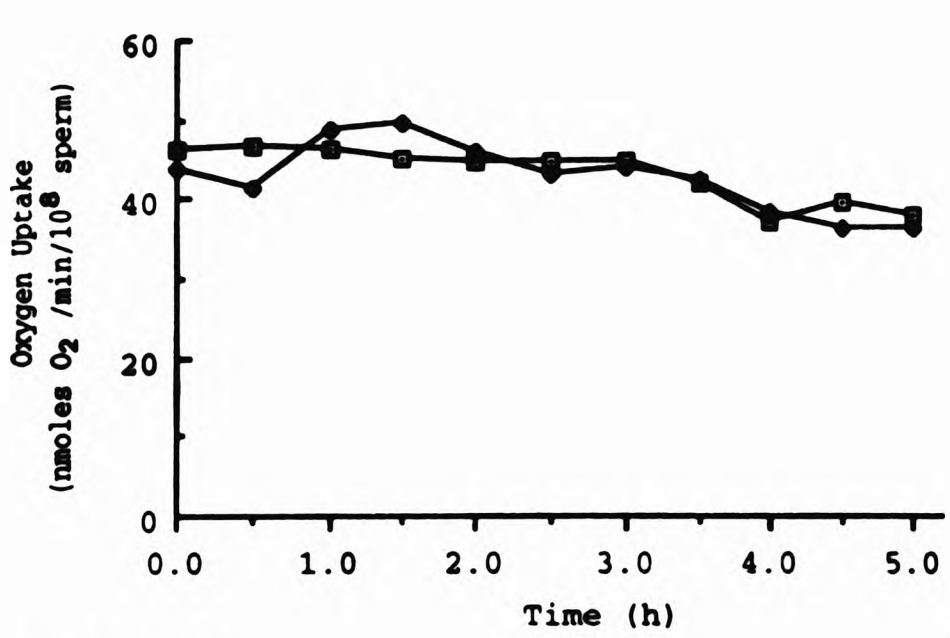
Fig. 6.IV. The Uptake and Hydrolysis of BCECF-AM by Spermatozoa.



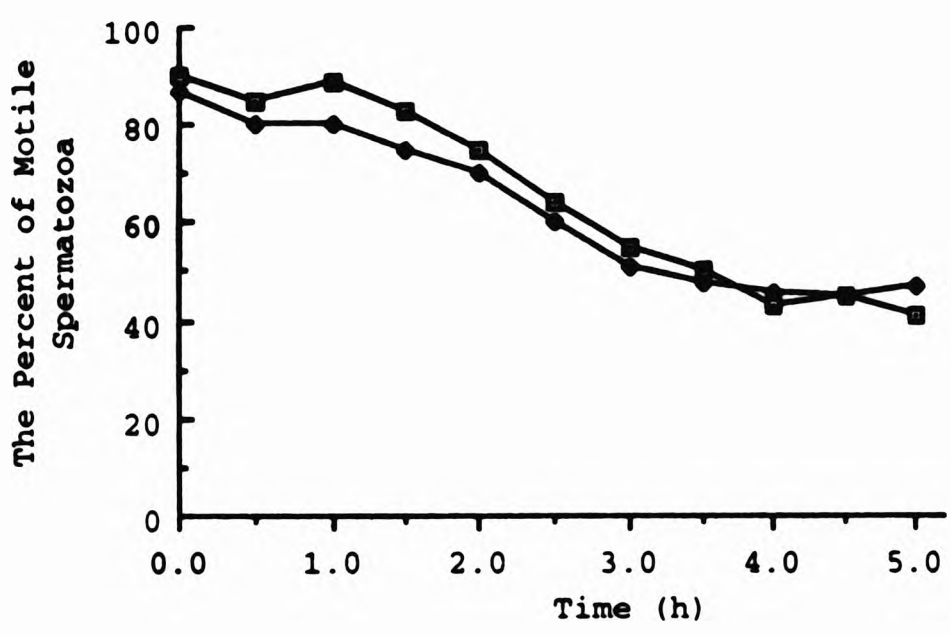
Washed intact, heat-treated or lysed spermatozoa (1-2 x10^8 spermatozoa/cm^3) were loaded with BCECF-AM (5µM). At regular intervals samples were removed and fluorescence measured as described in Materials and Methods. Results are expressed as a % of the maximum fluorescence. (n=6).

Fig. 6.V. The Effect of BCECF-AM on the Oxygen Uptake and Motility of Spermatozoa.

A. Oxygen Uptake



B. Motility



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated in ASW containing 5 μM BCECF-AM at 10°C. At the time points indicated 3 cm^3 aliquots were removed and both O_2 uptake and motility measured, as described in Materials and Methods. Values are means ± SD (n=6).

6.2.2. Measurement of the Intracellular pH of Spermatozoa from *Fucus serratus*.

Using the fluorescent indicator BCECF it can be seen that spermatozoa of *Fucus serratus* incubated in ASW at 10°C had an intracellular pH of 8.0 ± 0.1 (Fig. 6.VI). It has already been shown that the ionic composition and pH of ASW affects both motility and respiration of these spermatozoa. To investigate whether these parameters had an effect on the pHi of spermatozoa this pH was measured at various ASW compositions and pH values. The pHi was measured in ASW, ONaASW and OCaASW and found to be 0.6 of a pH unit and 0.7 of a pH unit lower in ONaASW and OCaASW respectively than in ASW (Table 6.1).

Table 6.I. The effect of the composition of ASW on the pHi of spermatozoa.

Composition of ASW	Extracellular pH	Intracellular pH
ASW	8.0	8.0 ± 0.1
ONaASW	8.0	7.4 ± 0.1
OCaASW	8.0	7.3 ± 0.1

Values are means ± SD (n = 3).

The pH of ASW was also important in regulating intracellular pH. When spermatozoa were diluted in acidic ASW their intracellular pH was also acidic however as the pH of ASW was raised from 5.0 → 8.0 then the pHi increased from 6.1→ 8.0 (Table 6.II).

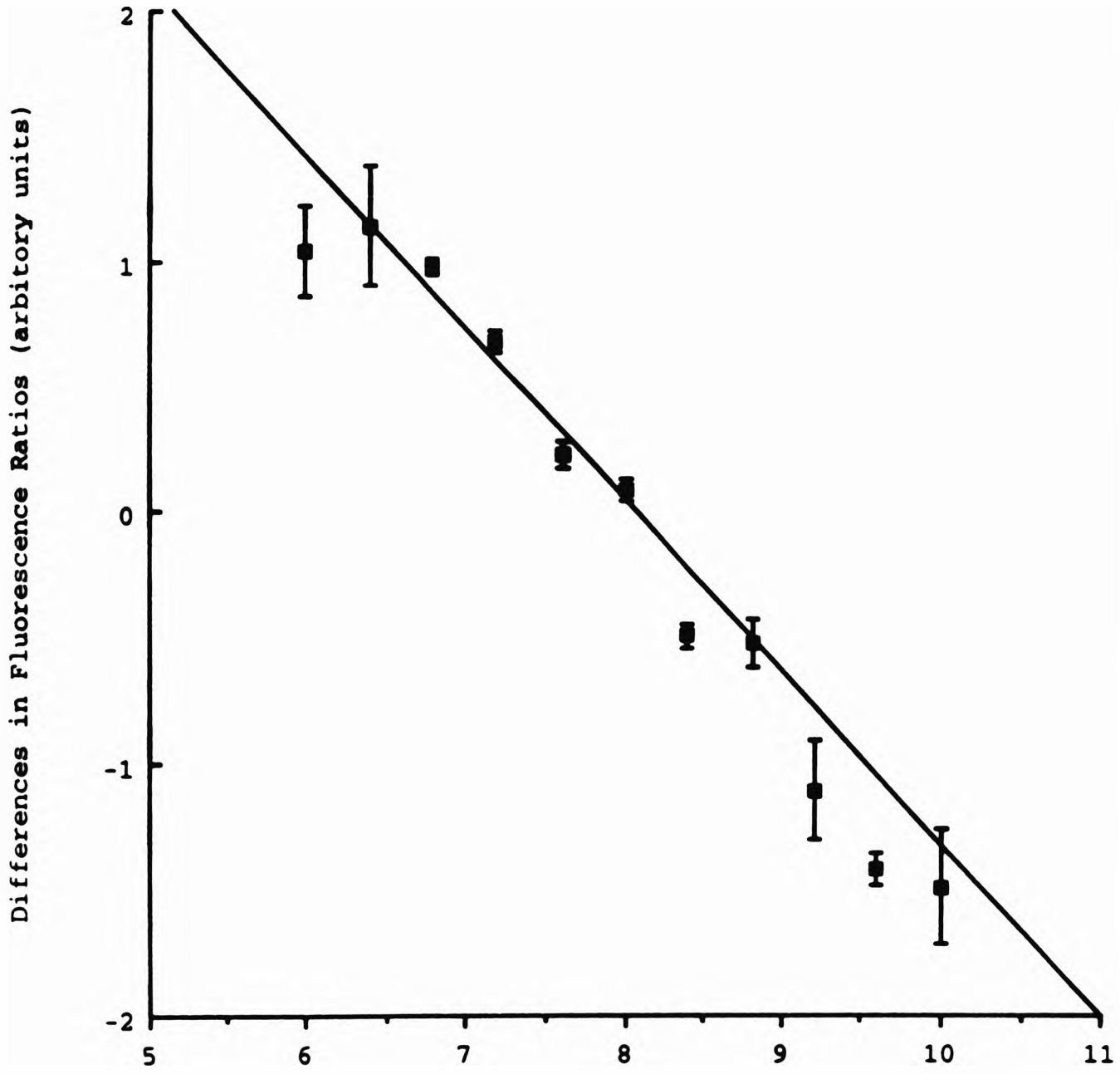
Table 6.II. The effect of extracellular pH on the pHi of spermatozoa.

Extracellular pH	Intracellular pH
5.0	6.1 ± 0.1
6.0	6.9 ± 0.2
7.0	7.3 ± 0.1
8.0	8.0 ± 0.1

Values are means ± SD (n = 3).

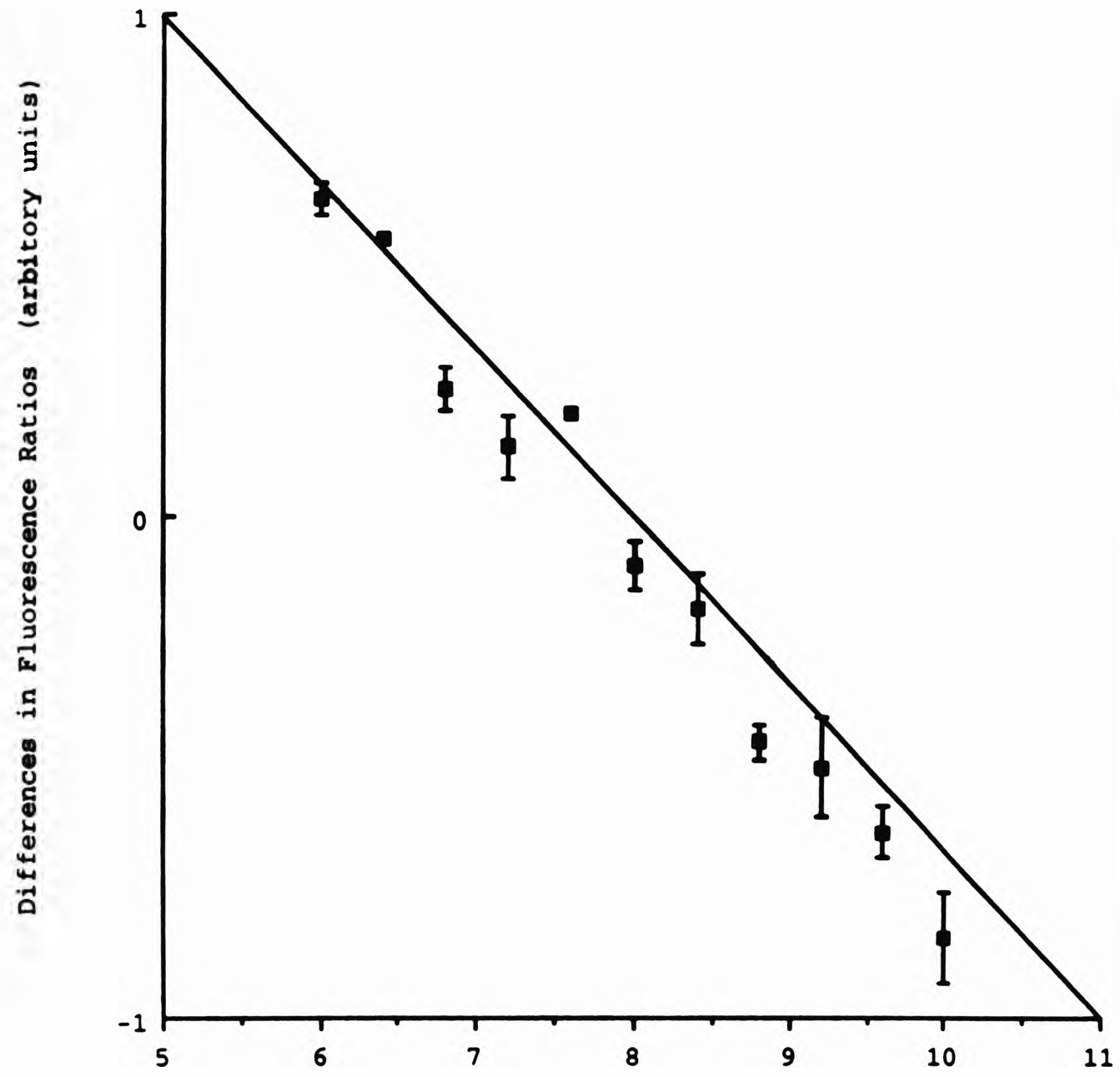
As previously mentioned intracellular pH increases in sea urchin sperm (Christen, *et al.*, 1982; 1983) when they are released into sea water and this change in pH, which has been associated with sperm activation, involves the Na^+/H^+ pump. Thus, it seemed appropriate to investigate the intracellular pH of spermatozoa from *Fucus serratus* incubated in ASW containing either an inhibitor or activator of this pump. Figures 6.VII and 6.VIII show the results of typical experiments. In the presence of the inhibitor, amiloride (final concentration 50 μM), pHi was lower by 0.3 units 7.7 ± 0.15 (n=3) than the pHi of spermatozoa suspended in ASW only (Figure 6.VII). Conversely, in the presence of the activator monensin (final concentration 0.1 μM) pHi increased by 0.8 units to 8.8 ± 0.05 (n=3) compared with the pHi of spermatozoa suspended in ASW in its absence (Figure 6.VIII).

Fig. 6.VI. **The Intracellular pH of Spermatozoa of Fucus serratus.**



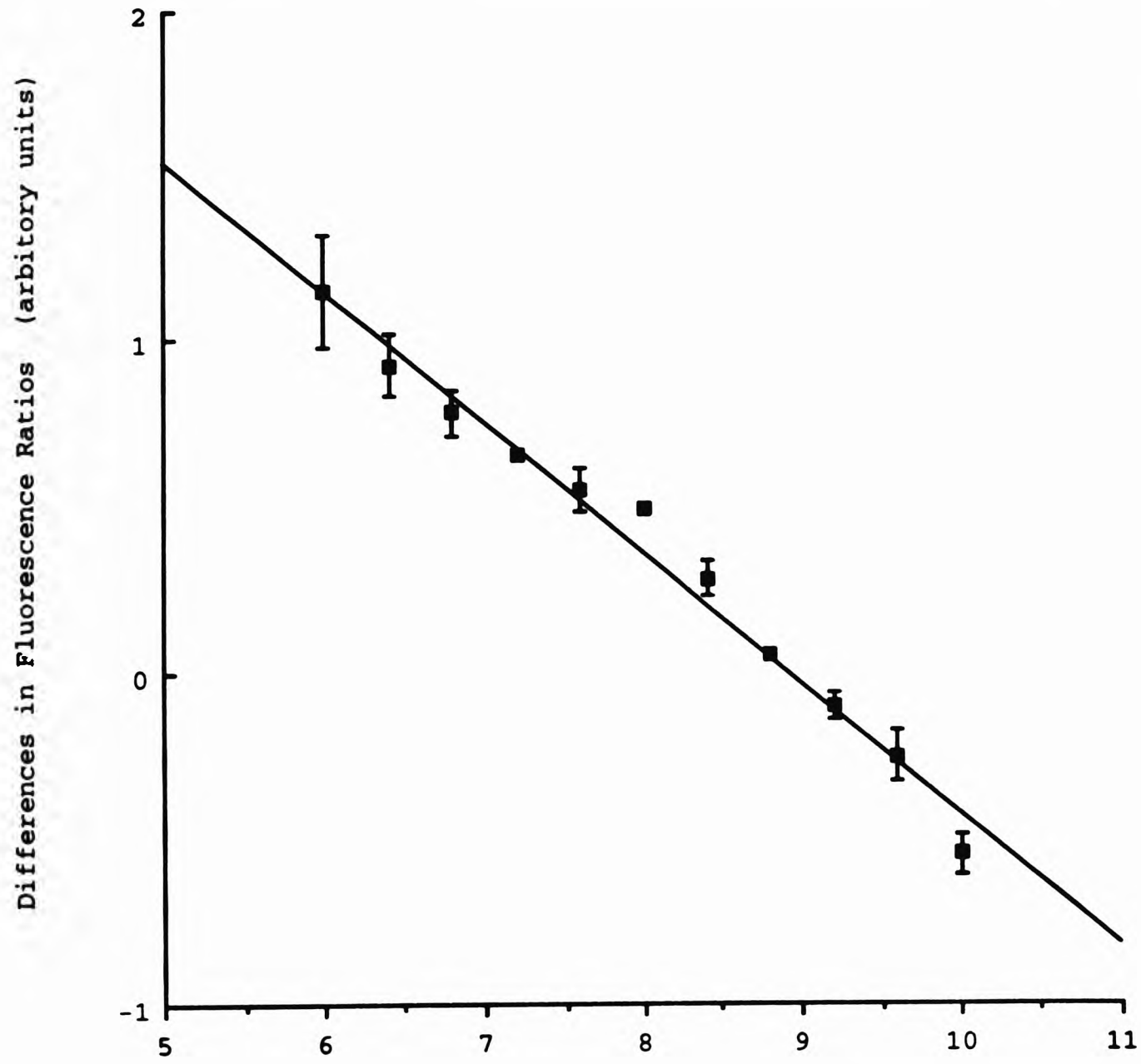
Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated in ASW containing 5 µM BCECF-AM for 3 hours at 10°C. After pelleting (2 cm^3 aliquots) by centrifugation (500g, 5 min) the spermatozoa were resuspended in 2 cm^3 ASW at the indicated pH. Fluorescence intensity was measured following excitation at both 490 nm and 465 nm, (emission was set at 550 nm), before and after the addition of 100 µg/cm^3 digitonin. The differences in fluorescence intensity ratios before and after digitonin addition were plotted as a function of external pH.

Fig. 6.VII. The Effect of Amiloride on the Intracellular pH of Spermatozoa.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated in ASW containing amiloride (50 µM) at 10°C for 15 minutes. Spermatozoa were then pelleted by centrifugation (2000 rpm, 5 min) and resuspended in ASW containing 5 µM BCECF-AM and incubated for 3 hours at 10°C. After pelleting the spermatozoa (2 cm^3 aliquots) by centrifugation (500g, 5 min) the spermatozoa were resuspended in 2 cm^3 ASW at the indicated pH. Fluorescence intensity was measured following excitation at 490 nm and 465 nm, (emission was set at 550 nm), before and after the addition of 100 µg/cm^3 digitonin. The differences in fluorescence intensity ratios before and after digitonin addition were plotted as a function of external pH.

Fig. 6.VIII. The Effect of Monensin on the Intracellular pH of Spermatozoa.



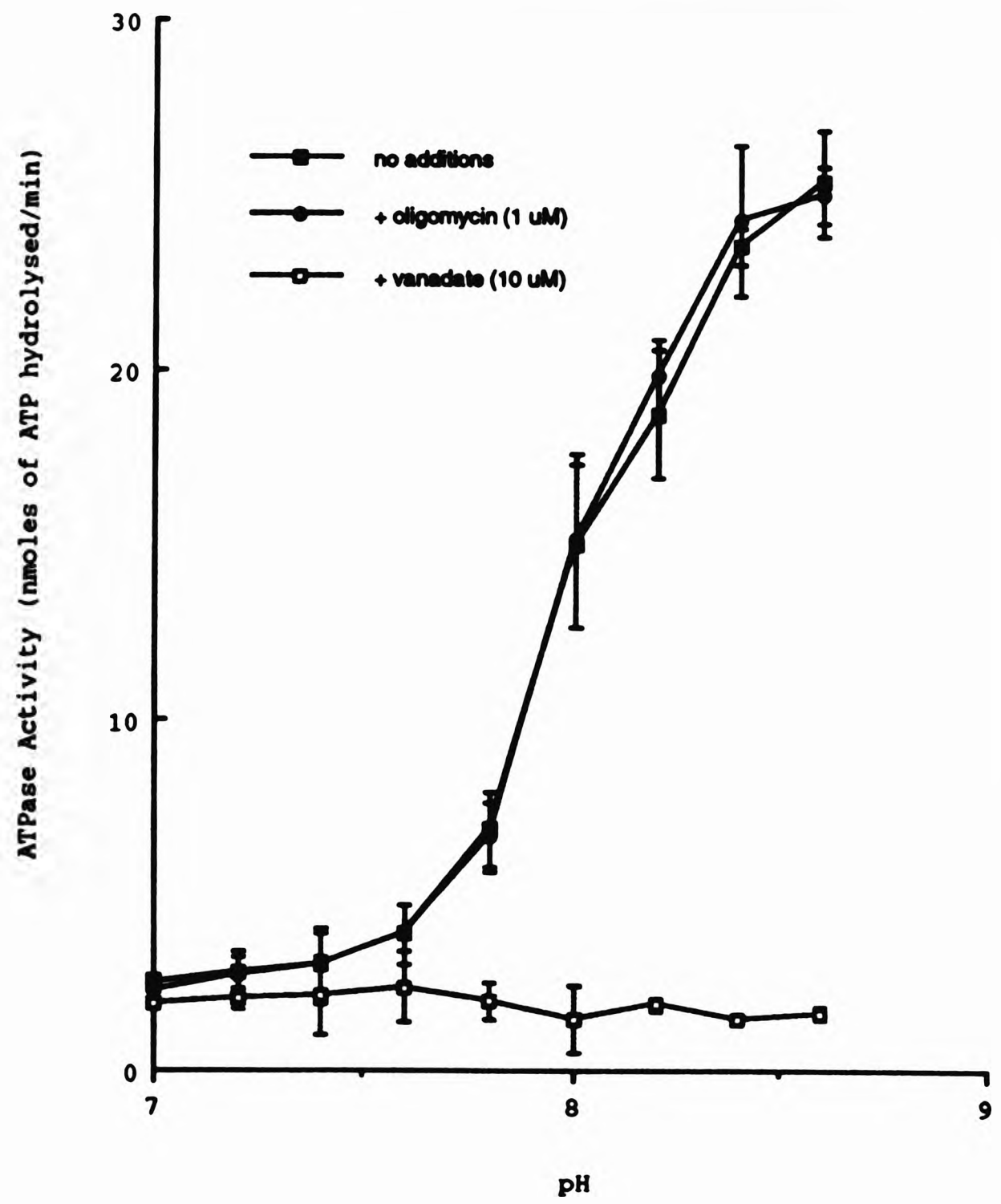
Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated in ASW containing monensin (0.1 µM) at 10°C for 15 minutes. Spermatozoa were then pelleted by centrifugation (2000 rpm, 5 min) and resuspended in ASW containing 5 µM BCECF-AM and incubated for 3 hours at 10°C. After pelleting the spermatozoa (2 cm^3 aliquots) by centrifugation (500g, 5 min) the spermatozoa were resuspended in 2 cm^3 ASW at the indicated pH. Fluorescence intensity was measured following excitation at 490 nm and 465 nm, (emission was set at 550 nm), before and after the addition of 100 µg/cm^3 digitonin. The differences in fluorescence intensity ratios before and after digitonin addition were plotted as a function of external pH.

6.3. The Effect of pH on the ATPase Activity of Permeabilized Spermatozoa.

In sea urchin sperm the activation of motility and respiration correlates with increased activity of dynein ATPase which is localised in the axoneme (Christen, *et al.*, 1983). It is probable that the ATP produced during respiration of spermatozoa from *Fucus serratus* is used for this activity. The ATPase activity of spermatozoa from *Fucus serratus* was estimated in sperm permeabilized with Triton X-100 (0.04% v/v) using the coupled assay of Gibbons and colleagues (1978) described in the Materials and Methods section. As shown in Figure 6.IX the ATPase activity of spermatozoa increased greatly when the pH of the incubating media was increased from 7.0 to 8.4 and the results show that the transition in activity occurs over a few tenths of a pH unit. Other ATPases, e.g. mitochondrial ATPase and Na^+/K^+ ATPase may also be present in these spermatozoa and contributing to the ATPase activity shown in Figure 6.IX. In order to ascertain the activities of individual ATPases assays were performed in the presence of known inhibitors of each one. The addition of oligomycin (1 μM) to the spermatozoa incubated at 10°C had no effect on the ATPase activity, indicating that mitochondrial ATPase was not contributing to the activity measured (Figure 6.IX). However the addition of vanadate (10 μM), an inhibitor of dynein ATPase, (Gibbons, *et al*, 1978; Okuno, 1980; Okuno and Brokaw, 1981) eliminated nearly all ATPase activity (Figure 6.IX).

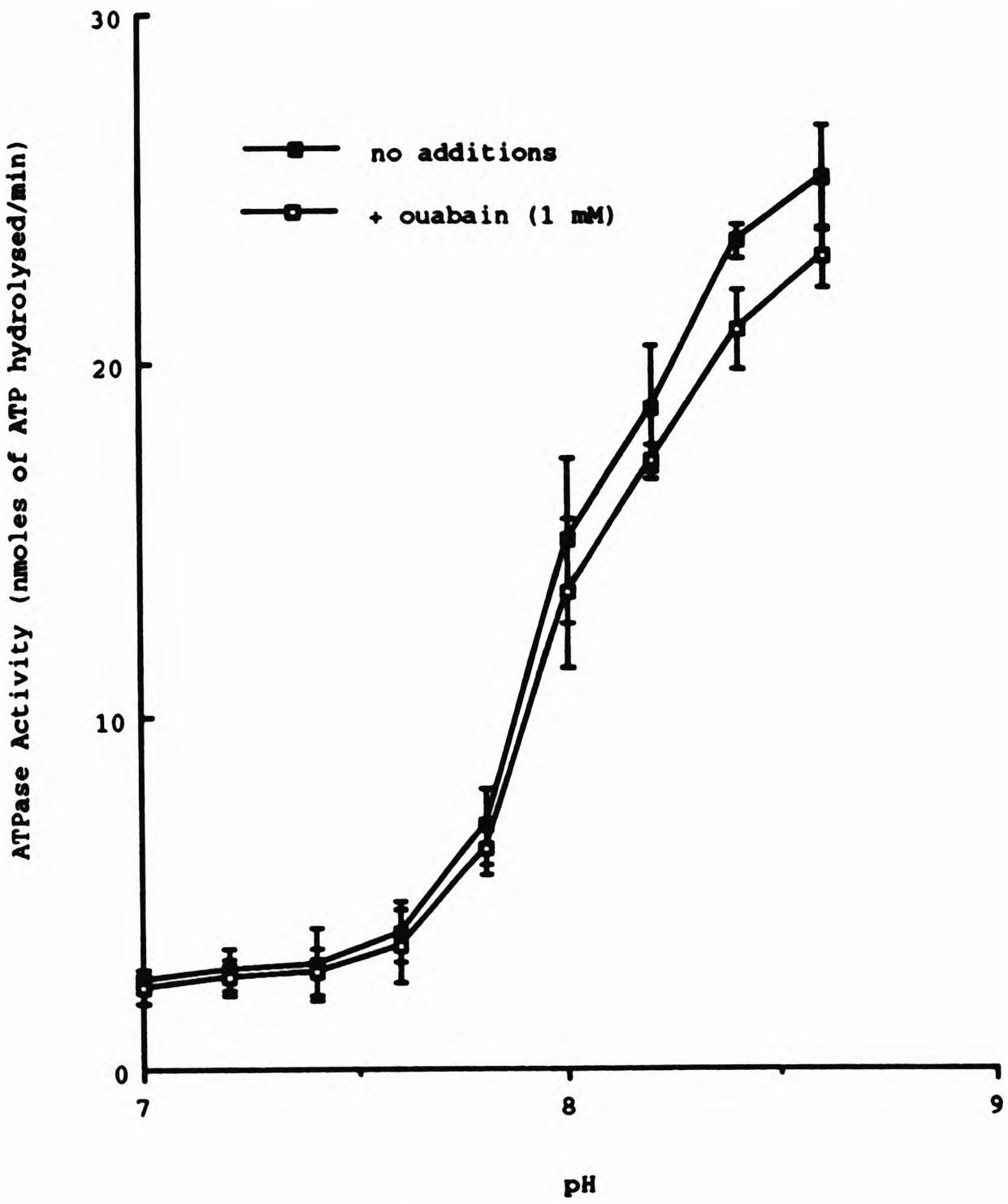
The addition of the Na^+/K^+ ATPase pump inhibitor ouabain (1 mM) resulted in less than 10% inhibition of ATPase activity, indicating that ATPase activity was primarily due to dynein ATPase (Figure 6.X).

Fig. 6.IX. The Effect of pH on the ATPase Activity of Spermatozoa I.



After permeabilization with 0.04% Triton X-100, spermatozoa were diluted into media of different pH and assayed for ATPase activity, as described in Materials and Methods, in the presence of oligomycin and vanadate.

Fig. 6.X. The Effect of pH on the ATPase Activity of Spermatozoa II.



After permeabilization with 0.04% Triton X-100, spermatozoa were diluted into media of different pH and assayed for ATPase activity, as described in Materials and Methods, in the presence of ouabain.

In order to determine whether dynein ATPase is directly linked to respiratory activity this enzyme was inactivated using the inhibitor vanadate. When added to a suspension of spermatozoa, vanadate inhibited respiration in the presence or absence of the respiration uncoupler FCCP and the inhibited rate of respiration was the same in both coupled and uncoupled sperm, see Table 6.III.

Table 6.III. The effect of vanadate on the O_2 uptake of spermatozoa.

Conditions	Mean O_2 uptake nmoles O_2/min/10^8 spermatozoa -FCCP	+FCCP
ASW	42.01 ± 4.44	
ASW + vanadate		
1 µM	17.39 ± 2.14 ***	18.15 ± 2.47
10 µM	11.16 ± 1.13 ***	11.77 ± 1.34
100 µM	3.97 ± 1.63 ***	4.82 ± 0.88

Values are means ± SD (n = 6).
*** P<0.001.

Thus, it appears that either respiration, even when it is uncoupled, requires a functional dynein ATPase activity or vanadate interferes directly with the respiratory chain.

6.4. The Respiratory Chain.

Oxidative phosphorylation is carried out by respiratory assemblies, as shown below, that are located in the inner membrane of mitochondria. Several inhibitors block the transfer of electrons through this transport chain, including rotenone, antimycin A and potassium cyanide.

$$\underset{\text{SITE I}}{\underset{\text{reductase}}{\text{NADH} \Rightarrow \text{NADH-Q}}} \Rightarrow \underset{\text{SITE II}}{\underset{\text{reductase}}{\text{Q} \Rightarrow \text{QH-cyt.c}}} \Rightarrow \text{ctc.c} \Rightarrow \underset{\text{SITE III}}{\underset{\text{oxidase}}{\text{cyt.}}} \Rightarrow O_2$$

Rotenone, a plant toxin, specifically inhibits electron transfer within the NADH-Q reductase complex, (site I), antimycin A, an antibiotic isolated from *Streptomyces griseus*, blocks electron flow between cytochromes *b* and c_1 (site II) and potassium cyanide inhibits the transfer of electrons from cytochrome *c* to molecular oxygen, (site III).

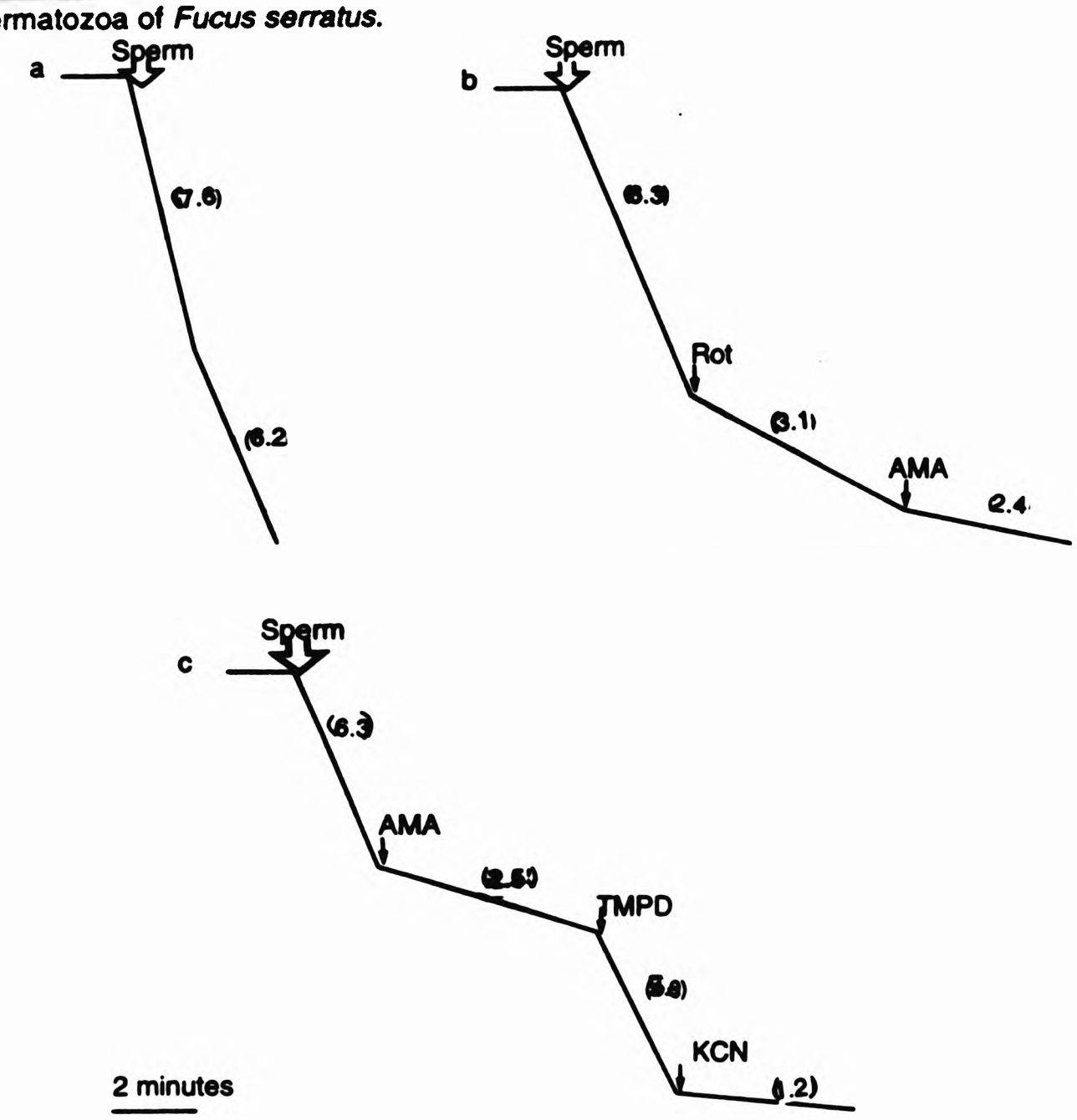
The effect of these inhibitors on the O_2 uptake of spermatozoa from *Fucus serratus*, was investigated. Addition of spermatozoa (final concentration of 2 x 10^8 spermatozoa/cm^3) to artificial sea water (ASW) resulted in a rapid decrease in oxygen levels in the ASW over the first 3 minutes (Fig. 6.XI). This initial rapid decline was followed by a slower but steady decline in oxygen (Fig. 6.XIa). The addition of rotenone or antimycin A and/ or potassium cyanide to a suspension of spermatozoa inhibited this O_2 uptake (Fig. 6.XIb). This inhibition of respiration caused by antimycin A was reversed by the addition of trimethyl-p-phenylene diamine (TMPD), an artificial electron donor which can supply electrons to cytochrome c, (Fig. 6.XIc), confirming that antimycin A was inhibiting electron transport in this system. This suggests that respiration of *Fucus serratus* spermatozoa involves electron transport through a

mitochondrial respiratory chain similar to that documented for mammalian and sea urchin spermatozoa.

The respiration of spermatozoa from *Fucus serratus* was also inhibited by oligomycin and this was reversed by 2, 4-dinitrophenol (DNP) (Figure 6.XIIa). As DNP is an uncoupler of oxidative phosphorylation these results suggest that electron transport in the spermatozoa is coupled with the phosphorylation of ADP.

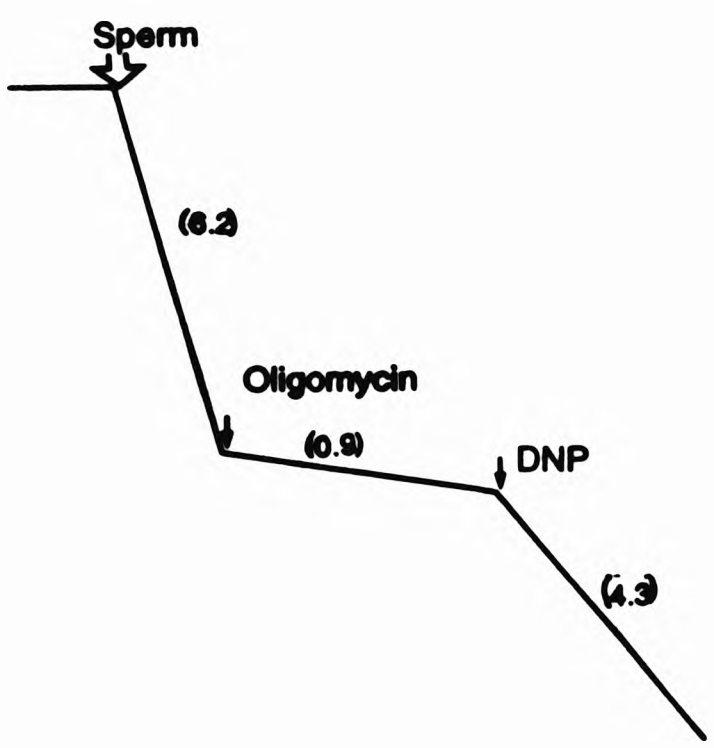
The rate of respiration in dense suspensions of spermatozoa, (Fig. 6.XIIIa 1) was lower than that in diluted suspensions, (Fig. 6.XIIIb 1). On the addition of DNP to dense suspensions of sperm (3 x 10^8 spermatozoa/cm^3) the rate of respiration was enhanced by 40 ± 5% (Figs. 6.XIIIa 1 & 2), whereas the addition of DNP to dilute suspensions of spermatozoa (2 x 10^8 spermatozoa/cm^3) only resulted in a enhancement of the respiratory rate by 8.5 ± 1.2% (Fig. 6.XIIIb 1 & 2). The spermatozoa in these experiments were also examined under a microscope, 90% of the spermatozoa were motile and were swimming vigorously.

Figure 6.XI. The effect of rotenone, antimycin A and cyanide on the respiration of spermatozoa of *Fucus serratus*.



Changes in oxygen levels in suspensions of spermatozoa of *Fucus serratus* in the presence of various inhibitors.
⇓ indicates sperm addition (final concentration was 2 x 10^8 spermatozoa/cm^3).
↓ indicates the addition of (1) rotenone (Rot, final concentration: 50μM).
(2) antimycin A (AMA, final concentration of 0.1μM).
(3) potassium cyanide (KCN, final concentration of 0.1 mM).
(4) TMPD (final concentration of 50μM).
The figures in brackets are respiration rates expressed as nmole O_2/min/10^8 spermatozoa. Traces shown in the figure are the average derived from the results of at least five experiments.

Figure 6.XII. The effect of oligomycin and DNP on the respiration of spermatozoa of *Fucus serratus*.



Change in oxygen levels in spermatozoa in the presence of oligomycin and an uncoupling agent.

⇓ indicates sperm addition (final concentration; $2x10^8$ spermatozoa/cm^3).

↓ indicates the addition of (1) oligomycin (final concentration: 50μM).
(2) 2, 4-dinitrophenol (DNP, final concentration: 50μM).

The figures in brackets are respiration rates expressed as nmole O_2/min/10^8 spermatozoa. Traces shown in this figure are the average of at least five experiments.

Figure 6.XIII. The effect of DNP on the respiration rates of densely packed and dilute suspensions of spermatozoa.

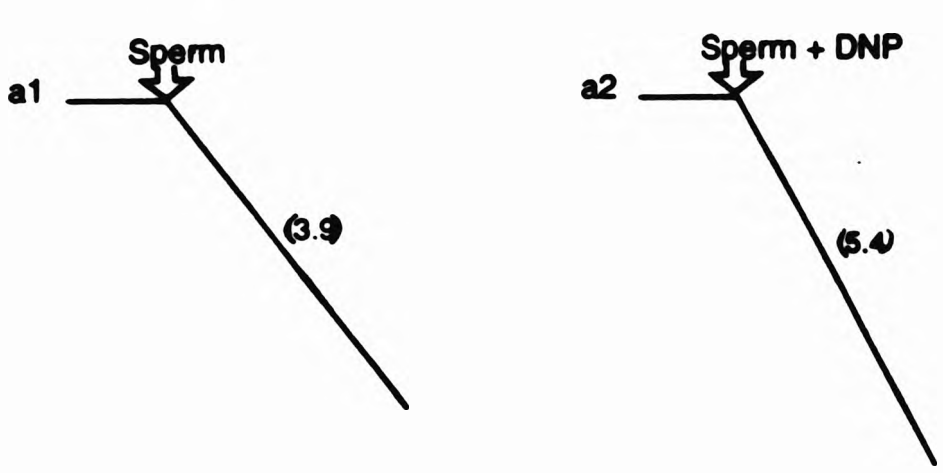


Fig. 6.XIa Dense Spermatozoa

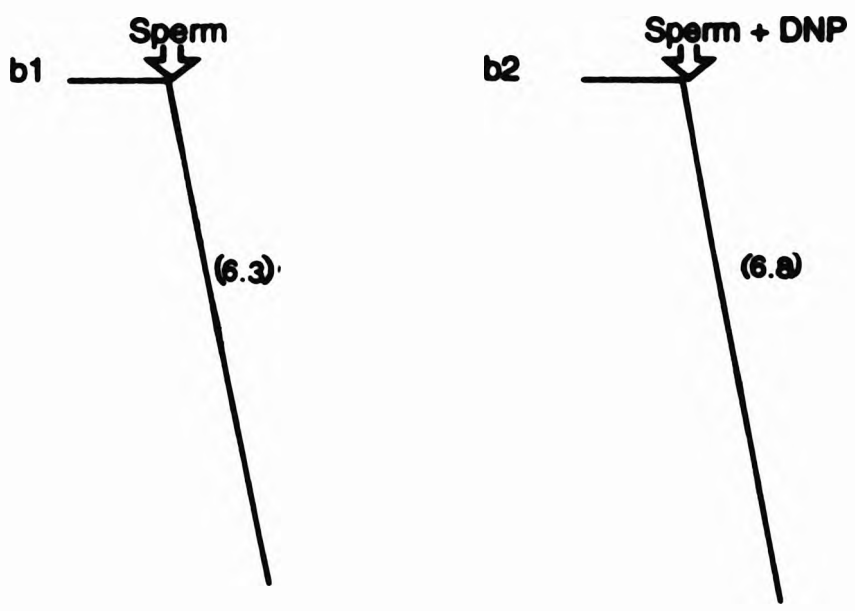


Fig. 6.XIb Diluted Spermatozoa

Change in oxygen levels in sperm suspensions in the presence of DNP.
Figure 6.XIa and 6.XIb shows the O_2 decrease in dense and diluted sperm suspensions respectively.
⇓ indicates the addition of spermatozoa (final concentration: dense suspensions 3 $x10^8$ spermatozoa/cm^3; diluted suspensions 2 x 10^8 spermatozoa/cm^3). DNP (final concentration: 50µl) was added to the sperm suspension from the start of the run.
The figures in brackets are respiration rates expressed as nmole O_2/min/10^8 spermatozoa. Traces shown in the figure are the average derived from the results of at least five experiments.

Many systems involved in sperm motility require ATP and this energy-dependence in spermatozoa from *Fucus serratus* was tested by measuring the percentage of motile sperm after incubation with an inhibitor of oxidative phosphorylation, antimycin A. This inhibitor rapidly causes depletion of active sea urchin sperm ATP levels (Christen, *et al.*, 1983). Spermatozoa from *Fucus serratus* were incubated in ASW with or without antimycin A (2 µM).

Table 6.IV. The effect of ATP depletion on motility.

Additions to ASW	Percent of Motile Spermatozoa
-	85 ± 4
antimycin A	33 ± 0.5 ***

Values are means ± SD (n=10).
*** P<0.001.

Table 6.IV shows that after depletion of intracellular ATP the percentage of motile spermatozoa was significantly reduced (61%).

6.5. Second Messengers.

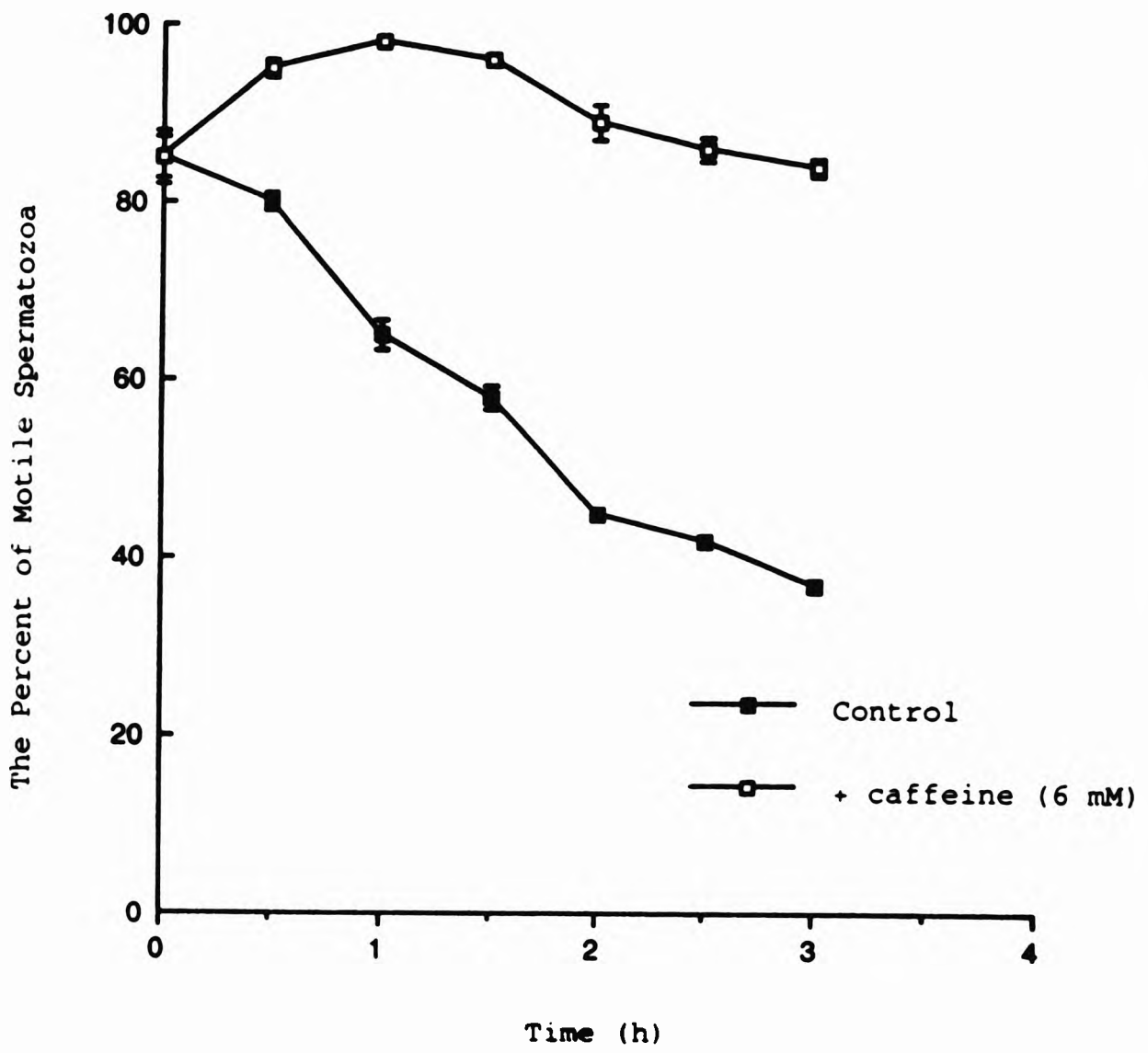
6.5.1. Adenosine 3',5'-cyclic Monophosphate.

A regulatory role of adenosine 3',5'-cyclic monophosphate (cAMP) on axonemal motility has been demonstrated with reactivated mammalian sperm models (Lindemann, 1978; Mohri and Yanagimachi, 1980). Cyclic nucleotides have also been shown to play a role in regulating the motility of mature mammalian spermatozoa (Hoskins and Casillas, 1975). For example, the addition of either cAMP phosphodiesterase inhibitors or dibutyryl cAMP (dbcAMP) to bovine epididymal spermatozoa (Hoskins and Casillas, 1975) and human ejaculated spermatozoa (Schoenfield, *et al.*, 1973) have been shown to stimulate their motility.

6.5.1.1. The Effect of Phosphodiesterase Inhibitors.

The addition of 6 mM caffeine, a cAMP phosphodiesterase inhibitor (Schoenfield, *et al.*, 1973), to spermatozoa from *Fucus serratus* increases motility from 85 ± 3.0% to > 98 ± 0.5% (n=6). The results in Figure 6.XIV show that the presence of caffeine in the incubating ASW not only stimulates motility but also enables spermatozoa to maintain this activated level of motion over a period of 3 hours, whereas in the absence of caffeine sperm motility falls to 25 ± 6.0% over the same time period. Caffeine (6 mM) also stimulates the respiration rate of spermatozoa. O_2 uptake increased significantly ($P<0.001$) from 46.35 ± 1.88 to 84.95 ± 3.50 nmoles O_2/min/10^8 spermatozoa (n = 6) after a 15 minute incubation period.

Fig. 6.XIV. The Effect of Caffeine on the Motility of Spermatozoa.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated at 10°C in ASW containing 6 mM caffeine. At the indicated time intervals 10 µl samples were removed and motility was quantified by videomicroscopy. The values are means ± SD (n=6).
*** P<0.001

Another cAMP phosphodiesterase inhibitor, theophylline has been shown to induce vigorous motility when added to epididymal bovine spermatozoa (Hoskins, *et al.*, 1974). Spermatozoa of *Fucus serratus* were incubated in ASW containing various concentrations of theophylline for 10 minutes then examined microscopically to measure motility. The results are shown in Table 6.V.

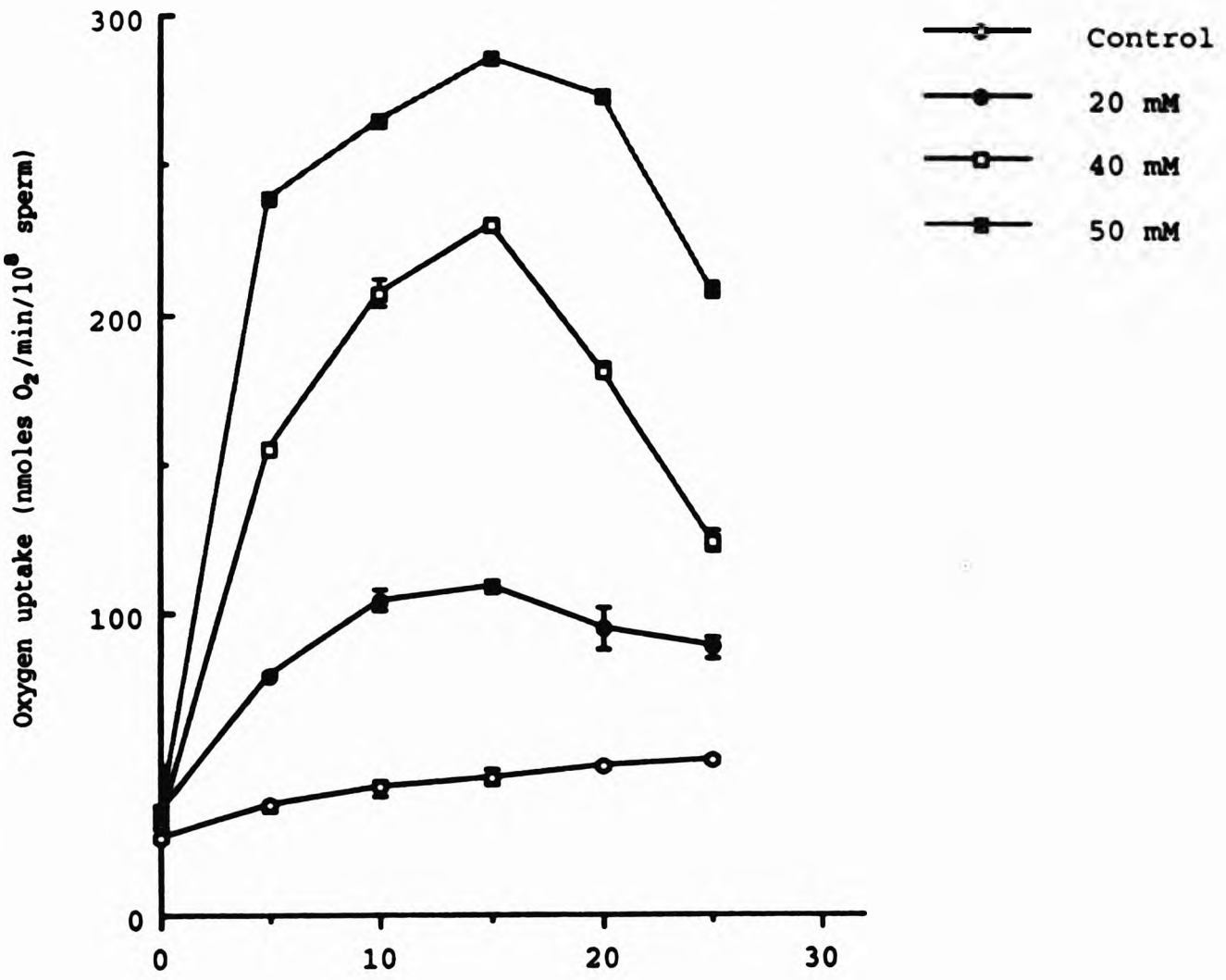
Table 6.V. The effect of theophylline on the motility of spermatozoa.

Concentration of theophylline (mM)	Percent of Motile Spermatozoa
0	81 ± 2.5
10	89 ± 1.0
20	93 ± 1.0
40	96 ± 1.5
50	99 ± 1.0

Values are means ± SD (n = 10).

At a concentration of 50 mM, theophylline almost all of the cells are motile. Theophylline also stimulates O_2 uptake in spermatozoa of *Fucus serratus* at all concentrations studied with the peak stimulation of 140% occurring after incubating spermatozoa for 15 minutes in the presence of 50 mM theophylline (Fig. 6.XV). Even at lower concentrations of theophylline, for example 20 mM a significant increase (48%) in O_2 uptake over the same time is observed.

Fig. 6.XV. The Effect of Theophylline on the Oxygen uptake of Spermatozoa.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated at 10°C in ASW for 15 minutes with the indicated amounts of theophylline. O_2 uptake was measured as described in "Materials and Methods". The values are means ± SD (n=10).

6.5.1.2. The Effect of Dibutyryl cAMP.

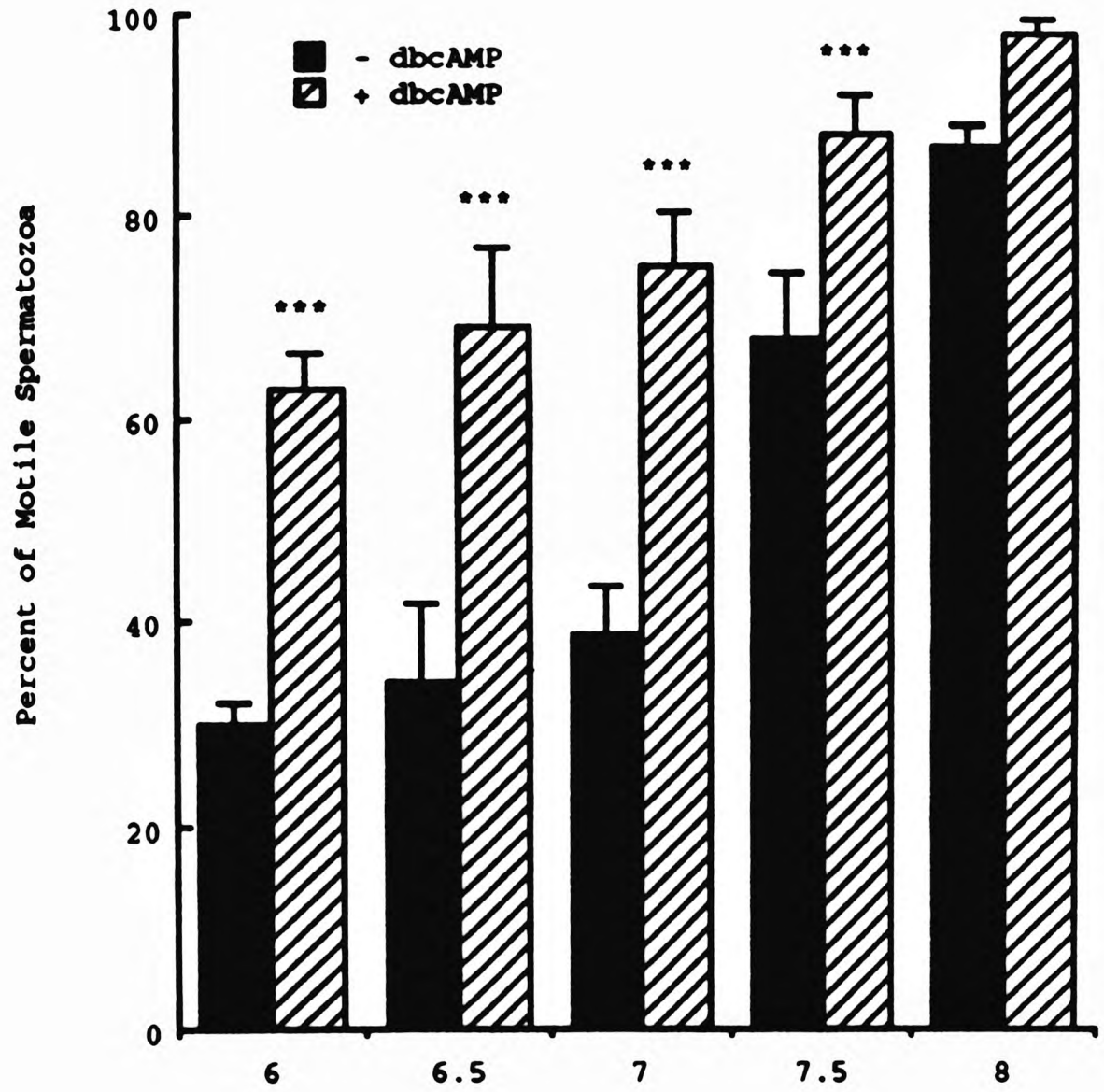
Cells are unable to take up exogenous cAMP but the cAMP analogue dibutyryl cAMP (dbcAMP) can cross the plasma membrane. The addition of dbcAMP to spermatozoa of *Fucus serratus* enhanced their motility. When 5 mM dbcAMP was included in the ASW the percentage motility of spermatozoa increased from 85 ± 2.0% to 99 ± 0.5% (n=6). Thus dbcAMP increases the amount of motile spermatozoa by approximately 15%. As previously shown (Table 4.II) the motility of spermatozoa is strongly dependent on extracellular pH. The effect of dbcAMP on the percent of motile sperm was determined over a narrow pH range. The results are given in Table 6.VI and Figure 6.XVI.

Table 6.VI. The effect of pH on the motility of spermatozoa.

pH	Percent of Motile Spermatozoa
6.0	30 ± 2.0
6.4	37 ± 5.0
6.8	42 ± 2.0
7.2	52 ± 2.0
7.6	69 ± 1.0
8.0	87 ± 3.0
8.4	80 ± 4.0
8.8	55 ± 5.0
9.2	31 ± 3.0

Values are means ± SD (n = 10).

Fig 6.XVI. The Effect of dbcAMP on pH-dependent Motility.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated at 10°C in ASW at the indicated pH ± 5 mM dbcAMP. Motility was quantified by videomicroscopy at 10 minutes. The values are means ± SD (n=10).
***P<0.001

The percent of motile spermatozoa is optimum at pH 8.0 and is substantially reduced when the extracellular pH is below 7.0.

The results in Figure 6.XVI show the striking change in the relationship between motility and pH when sperm were stimulated with 5 mM dbcAMP. Spermatozoa incubated in ASW at the lower pH range (6 → 7.5) exhibited greatly enhanced motility in the presence of dbcAMP. At pH 6.0 motility was stimulated by 110% but as the pH of the ASW was increased towards the optimum of pH 8.0 the extent of stimulation decreased to only 16%.

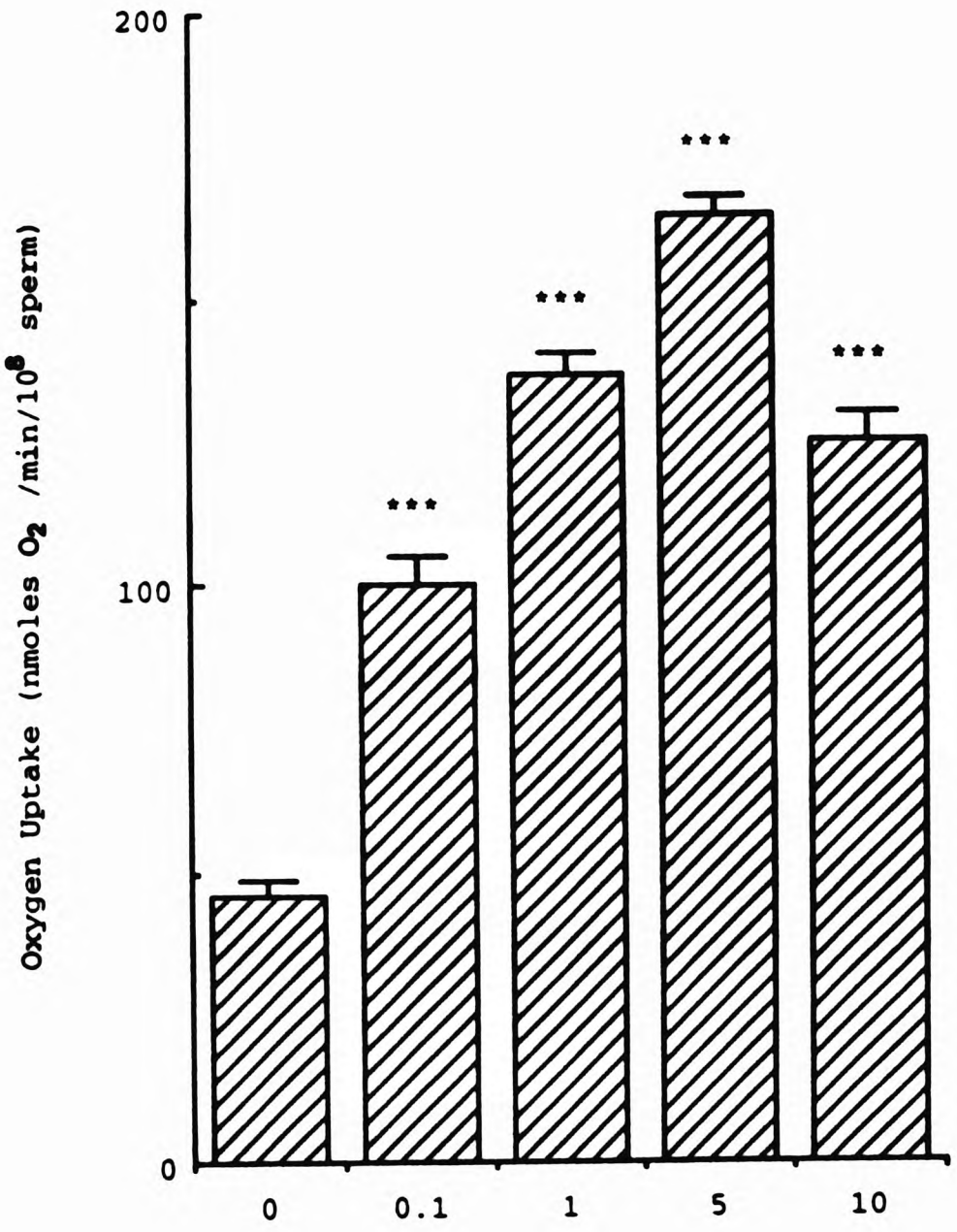
The addition of dbcAMP to suspensions of spermatozoa also resulted in an increase in the rate of respiration. The results in Figure 6.XVII show that in the presence of 5 mM dbcAMP (pH 8.0) O_2 uptake increased significantly ($P<0.001$) by 117% from 46.35 ± 2.88 to 100.46 ± 4.94 nmoles O_2/min/10^8 spermatozoa. Even at the relatively low concentration (0.1 mM) of dbcAMP O_2 uptake increased by 25% from 46.35 ± 2.88 to 58.14 ± 2.35 nmoles O_2/min/10^8 spermatozoa and this stimulation occurs almost immediately. The addition of 5 mM dbcAMP to a suspension of spermatozoa which had been incubated for 2 minutes produced an immediate rise in O_2 uptake from 14.31 ± 2.56 to 22.56 ± 1.26 nmoles O_2/min/10^8 spermatozoa (58% stimulation).

6.5.1.3. <u>The Effect of Forskolin.</u>

Forskolin has been shown to activate the motility of mammalian spermatozoa and it seems to employ cAMP as a second messenger (Garbers and Kopf, 1980; Tash and Means, 1982a & b; Brokaw, 1987). The addition of varying concentrations of this agent to a suspension of spermatozoa from *Fucus serratus* resulted in an elevation of O_2 uptake (Figure 6.XVIII). 100 µM, forskolin increased O_2 uptake from 45.18 ± 2.22 to 103.74 ± 2.22 nmoles O_2/min/10^8 spermatozoa.

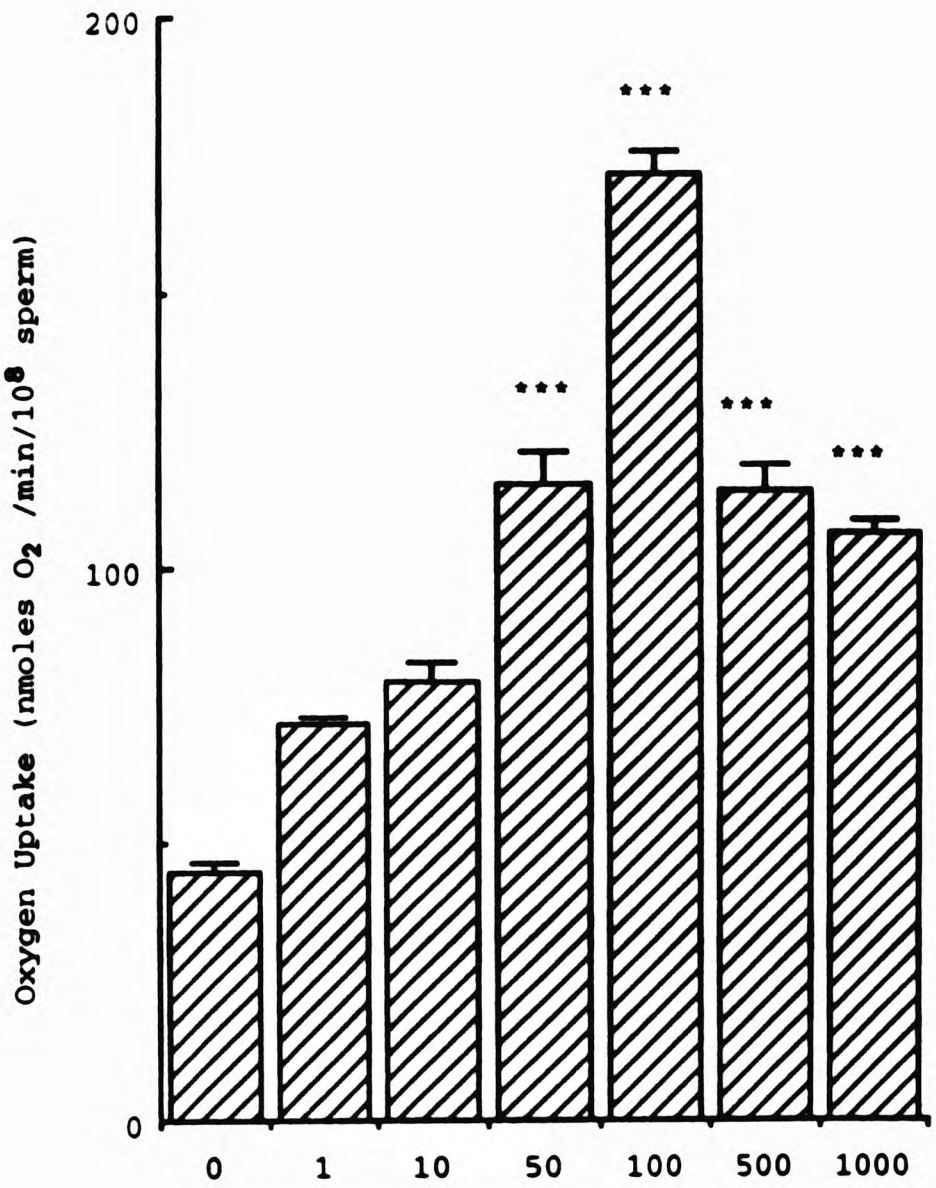
The results of these experiments with inhibitors of phosphodiesterase, cAMP analogues and other agents which affect cAMP levels demonstrate that the motility and respiration of spermatozoa from *Fucus serratus* can be influenced by modulators of the adenylate cyclase/cAMP second messenger system, thus implying a role for that system in sperm motility. Additionally, other second messenger systems may also be involved.

Fig. 6.XVII. The Effect of dbcAMP on the Oxygen Uptake of Spermatozoa.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated at 10°C in ASW for 15 minutes with the indicated amounts of dbcAMP. O_2 uptake was measured as described in "Materials and Methods". The values are means ± SD (n=10).
***P<0.001

Fig. 6.XVIII. The Effect of Forskolin on the Oxygen Uptake of Spermatozoa.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated at 10°C in ASW for 15 minutes with the indicated amounts of forskolin. O_2 uptake was measured as described in "Materials and Methods". The values are means ± SD (n=10).
***P<0.001

6.5.2. The Presence of Protein Kinase C in Spermatozoa.

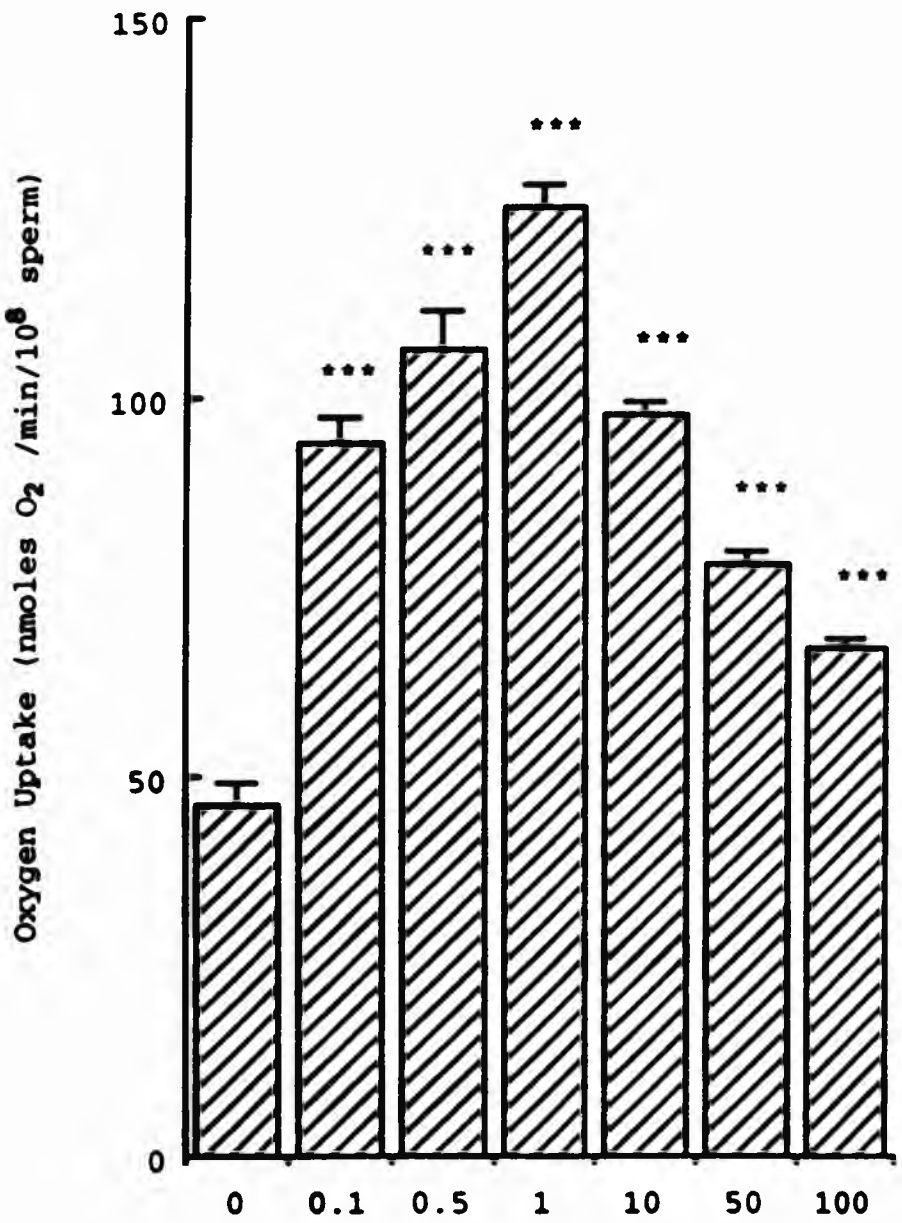
Calcium-dependent protein kinase C is activated by the second messenger diacylglycerol (De Jonge, *et al.*, 1991). Phorbol diesters are compounds that stimulate protein kinase C *in vivo* and *in vitro* (Castatagna, *et al.*, 1982), and the present experiments were performed using phorbol 12-myristate 13 acetate (PMA).

In preliminary experiments (n = 3) when 100 µM PMA, a concentration based on data for somatic cells (Castagna, *et al.*, 1982), was added to spermatozoa, no significant effect on O_2 uptake was observed although slight inhibition of sperm motility (10%) occurred. As these results were inconclusive lower concentrations of PMA were tested. Results in Figure 6.XIX show that when spermatozoa were incubated for 10 minutes in the presence of 1 nM PMA, a significant ($P<0.001$) increase in O_2 uptake (370%) occurred in comparison with untreated controls. PMA, at the same concentration also stimulated the percent of motile spermatozoa by 12% (from 81 ± 2% to 91 ± 1%), when compared with untreated controls. In other systems protein kinase C is dependent on Ca^{2+} for activity but PMA slightly stimulated O_2 uptake and motility of spermatozoa even when they were suspended in OCaASW. Upon the addition of PMA (1 nM), O_2 uptake rose from 15.78 ± 1.70 to 19.98 ± 2.35 nmoles O_2/min/10^8 spermatozoa (n=10) and motility increased by 15%, from 84 ± 1% to 97 ± 1% (n=10). Rink and colleagues (1983) have reported PKC mediated reactions in human platelets, which were stimulated by phorbol diesters but were Ca^{2+}-independent. The above results show that both Ca^{2+}-dependent and Ca^{2+}-independent PKC mediated reactions may be involved in the activation of spermatozoa from *Fucus serratus*.

To confirm this stimulatory effect of PMA on O_2 uptake an additional phorbol diester, 4 β-phorbol 12, 13 didecanoate (β-PDD), was tested for its effect on O_2

uptake. The concentrations of β-PDD selected were based on the previous results using PMA and the results are shown in Table 6.VII.

Fig. 6.XIX. The Effect of PMA on the Oxygen Uptake of Spermatozoa.



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated at 10°C in ASW for 15 minutes with the indicated amounts of PMA. O_2 uptake was measured as described in "Materials and Methods". The values are means ± SD (n=10).
***P<0.001

Table 6.VII. The effect of β-PDD on the O_2 uptake of spermatozoa.

Concentration of β-PDD nM	O_2 uptake nmoles O_2/min/10^8 spermatozoa
0	44.29 ± 2.06
0.1	92.18 ± 5.29 ***
1	162.31 ± 4.64 ***
10	117.15 ± 2.47 ***

Values are means ± SD (n = 6).
*** P<0.001.

The results in Table 6.VII show that 1 nM β-PDD stimulates O_2 uptake by 266%.

To further establish whether protein kinase C plays a role in the activation of sperm motility and respiration an inhibitor of protein kinase C, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) (Rotem, *et al.*, 1990a), was tested for its effect on motility and respiration of spermatozoa incubated in ASW (Figure 6.XX and Table 6.VIII).

Table 6.VIII. The effect of H-7 on the motility of spermatozoa.

Concentration of H-7 $\mu g/cm^3$	Percent of Motile Spermatozoa
0	84 ± 2.5
5	73 ± 5.0 **
10	60 ± 4.9 ***
15	37 ± 5.9 ***
20	24 ± 5.5 ***
30	19 ± 1.5 ***

Values are means ± SD (n = 6).
** P<0.01.
*** P<0.001.

It can be seen from Table 6.VIII that the motility of spermatozoa incubated in ASW was inhibited by H-7 and the greatest degree of inhibition (77%), that was observed, occurred at 30 $\mu g/cm^3$. At a level of 30$\mu g/cm^3$, H-7 also inhibited the O_2 uptake of spermatozoa suspended in ASW (88%) (Figure 6.XX).

As H-7 is an inhibitor of PKC it seemed appropriate to investigate whether this compound inhibited motility and respiration after the spermatozoa were stimulated with the phorbol diester PMA. A suspension of spermatozoa were incubated with H-7 (20 ug/cm^3) for 10 minutes, then PMA, at a final concentration of 1 nM, was added and the suspension incubated for a further 10 minutes after which time motility and O_2 uptake were measured (Table 6.IX).

Table 6.IX. The effect of H-7 on PMA stimulated spermatozoa.

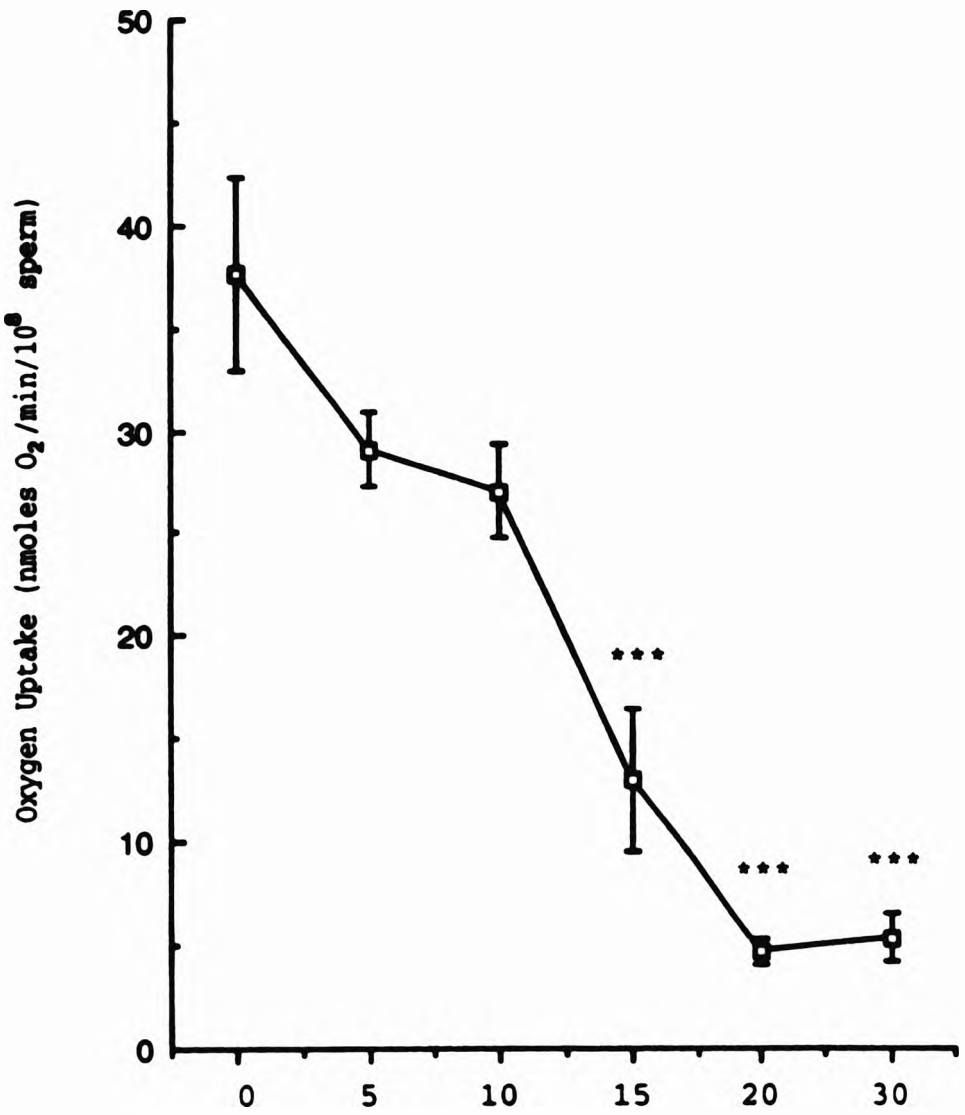
Conditions	Percent of Motile Spermatozoa	O_2 uptake nmoles O_2/min/10^8 sperm
ASW	84 ± 2.5	44.24 ± 3.06
+ PMA	99 ± 1.0	125.06 ± 3.57 ***
+ PMA & H-7	28 ± 1.5 ***	7.64 ± 2.35 ***

Values are means ± SD (n=10)
*** P<0.001

The results in the above table show that H-7 inhibits both motility and respiration of spermatozoa even in the presence of an activator of PKC, PMA, which had previously been shown to stimulate both the motility and respiration of spermatozoa of *Fucus serratus* and the degree of inhibition was as great as when H-7 alone was added to spermatozoa.

In conclusion, it would appear that PKC is the target for stimulation by PMA and inhibition by H-7 and that PKC may play a role in the enhancement of motility and respiration of *Fucus serratus* spermatozoa.

Fig. 6.XX. The Effect of H-7 on the Oxygen Uptake of Spermatozoa



Washed spermatozoa (1-2x10^8 spermatozoa/cm^3) were incubated at 10°C in ASW for 15 minutes with the indicated amounts of H-7. O_2 uptake was measured as described in "Materials and Methods". The values are means ± SD (n=10).
***P<0.001

6.5.3. External Calcium.

As already demonstrated calcium is an ion that appears to play a critical role in initiating sperm motility. Spermatozoa incubated in ASW minus Ca^{2+} (0CaASW) have 70% fewer motile sperm and an O_2 uptake 67% lower than spermatozoa incubated in ASW (Table 4.III). The re-introduction of calcium into the ASW results in an increase in both the motility and O_2 uptake of these spermatozoa. Ca^{2+} (10 mM) stimulates sperm motility by 89% and O_2 uptake by 89%.

6.5.4. Intracellular Calcium.

As exogenous calcium stimulates O_2 uptake and motility in spermatozoa of *Fucus serratus*, it seemed that changes in intracellular free calcium concentration might play a role in the initiation of sperm motility.

6.5.4.1. The Effect of EGTA.

Unbound Ca^{2+} in a cell can be decreased to nanomolar concentrations by introducing a Ca^{2+} specific chelator such as EGTA. Therefore, any processes mediated by a rise in the cytosolic level of Ca^{2+}, such as has been postulated for the initiation of motility in spermatozoa should be blocked with such a chelator. The addition of varying concentrations of EGTA to suspensions of spermatozoa resulted in the inhibition of both motility and respiration. The results in Table 6.X show that 10 mM EGTA inhibited O_2 uptake by 70% from 42.98 ± 2.31 to 12.96 ± 2.04 nmoles O_2/min/10^8 spermatozoa and motile spermatozoa by 82%. Even lower concentrations of EGTA caused substantial inhibition; 0.1 mM inhibited O_2 uptake by 26% and sperm motility by 61%.

Table 6.X. The effect of EGTA on the motility and O_2 uptake of spermatozoa.

Concentration of EGTA mM	Percent of Motile Spermatozoa	Mean O_2 Uptake nmoles O_2/min/10^8 sperm
0	94 ± 2.3	42.98 ± 2.31
0.01	75 ± 2.1 ***	33.05 ± 3.72
0.1	37 ± 4.9 ***	32.01 ± 2.86
1	28 ± 4.0 ***	24.19 ± 2.56 ***
10	17 ± 1.4 ***	12.96 ± 2.04 ***

Values are means ± SD (n = 10).
*** P<0.001.

The respiration rate of spermatozoa from *Fucus serratus* incubated in OCaASW in the presence of 10 mM EGTA was very low [3.07 ± 1.42 nmoles O_2/min/10^8 spermatozoa (n = 6)] and almost all the sperm were immotile [<2 ± 1% motility (n = 6)]. After centrifugation (600 x g for 10 minutes) to remove excess EGTA, the spermatozoa were resuspended in normal ASW and their O_2 uptake measured, upon the re-introduction of Ca^{2+} the O_2 uptake of these spermatozoa increased by 188% to 8.86 ± 1.90 nmoles O_2/min/10^8 spermatozoa (n = 5) and the percent of motile sperm increased by 700% to 16 ± 1.4% (n = 5). Although, following the re-introduction of Ca^{2+} to sperm after their incubation with EGTA, both O_2 uptake and the percent of motile spermatozoa rise the levels do not achieve that seen when spermatozoa are incubated in ASW in the absence of EGTA. This implies that, at the concentration added, EGTA is still capable of chelating some of Ca^{2+} added to the spermatozoa.

These results indicate that Ca^{2+} affects both respiration and motility in spermatozoa of *Fucus serratus* and may play a role in the initiation of both these parameters when these spermatozoa are released into sea water.

6.5.4.2. The Effect of the Ca^{2+} Ionophore A23187.

Spermatozoa are insensitive to hormones and physiological agents, known to affect calcium fluxes in other cells (Hoskins and Casillas, 1975; Garbers and Kopf, 1980). The only way known to increase intracellular calcium levels in mammalian spermatozoa is through the effect of the calcium ionophore, A23187 (Reed and Lardy, 1972; Vijayaraghavan and Hoskins, 1989). As the previous results with Ca^{2+}-free ASW, EGTA and verapamil (see chapter 5) indicate that the intracellular concentration of Ca^{2+} may play a vital role in the onset of motility and respiration of spermatozoa of *Fucus serratus*, these parameters were measured in the presence of the ionophore, A23187. Spermatozoa, suspended in ASW, OCaASW and ONaASW, were incubated in the presence of A23187, at a final concentration of 10 µM, which had been shown to activate *Fucus* eggs (Brawley and Bell, 1987), for 10 minutes after which time samples were removed and both percentage motility and O_2 uptake were measured, the results are shown in Table 6.XI.

Table 6.XI. The effect of A23187 on the motility and O_2 uptake of spermatozoa.

Conditions	Percent of Motile Spermatozoa	Mean O_2 Uptake nmoles O_2/min/10^8 sperm
ASW	84.0 ± 1.4	41.55 ± 2.37
+ A23187	99.0 ± 0.8 ***	80.77 ± 1.77 ***
OCaASW	23.0 ± 2.6	15.37 ± 1.54
+ A23187	28.0 ± 2.9	18.25 ± 1.79
ONaASW	1.0 ± 0.9	8.12 ± 0.58
+ A23187	87.0 ± 6.2***	25.00 ± 2.61 ***

Values are means ± SD (n=10)
*** P<0.001.

The presence of the calcium ionophore stimulated both motility and O_2 uptake of spermatozoa suspended in all three types of sea water however, the degree of stimulation differed. The O_2 uptake of spermatozoa suspended in ASW was enhanced by 94% and these spermatozoa had virtually 100% motility. When added to spermatozoa suspended in OCaASW, A23187 did not significantly increase O_2 uptake nor sperm motility. At a concentration of 10 µM, A23187 stimulated the O_2 uptake of spermatozoa, suspended in ONaASW, by 208% and the percent of motile spermatozoa increased to a level equal to that seen in spermatozoa suspended in ASW.

These data demonstrate that spermatozoa were sensitive to the calcium ionophore, A23187. The reduced response to A23187 by spermatozoa suspended in ASW compared to spermatozoa suspended in ONaASW may be due to the presence of a Na^+/Ca^{2+} antiporter, which exchanges intracellular Ca^{2+} for extracellular Na^+, this exchange would be eliminated in Na^+-free ASW.

6.5.4.3. <u>Measuring Intracellular Calcium.</u>

The introduction of fluorophores, for example Quin-2 and Fura-2, to quantitate intracellular free calcium levels has made studying the effects of intracellular calcium feasible in several somatic cells (Tsien, 1980; 1981; Tsien, *et al.*, 1982; Rink and Pozzan, 1985). These fluorophores have also been used to measure the concentration of intracellular free calcium in bovine epididymal spermatozoa (Vijayaraghavan and Hoskins, 1989), and in sea urchin spermatozoa (Trimmer, *et al.*, 1986). Fura-2 is available as a membrane-permeable acetoxymethylester (Fura-2-AM) in which form it readily crosses the plasma membrane and enters the cytoplasm, where it is hydrolysed by intracellular esterases to the parent free acid (Fura-2), see Figure 6.XXI. This acid form is membrane impermeable and thus becomes trapped

within the cytoplasm and can be used for the direct measurement of intracellular free calcium concentrations $[Ca^{2+}]i$ (Mazorow and Millar, 1990). The fluorescent properties of Fura-2 are such that the ratio of fluorescence intensity at excitation values of 350 and 385 nm, with emission set at 500 nm, is Ca^{2+} dependent (Grynkiewicz, *et al.*, 1985).

6.5.4.4. <u>Fluorescence Microscopy.</u>

The uptake of Fura-2-AM was visualised using a Zenith fluorescence microscope, as described in Materials and Methods. The photographs in Figure 6.XXII show that Fura-2-AM was taken up into the body of the spermatozoa of *Fucus serratus*.

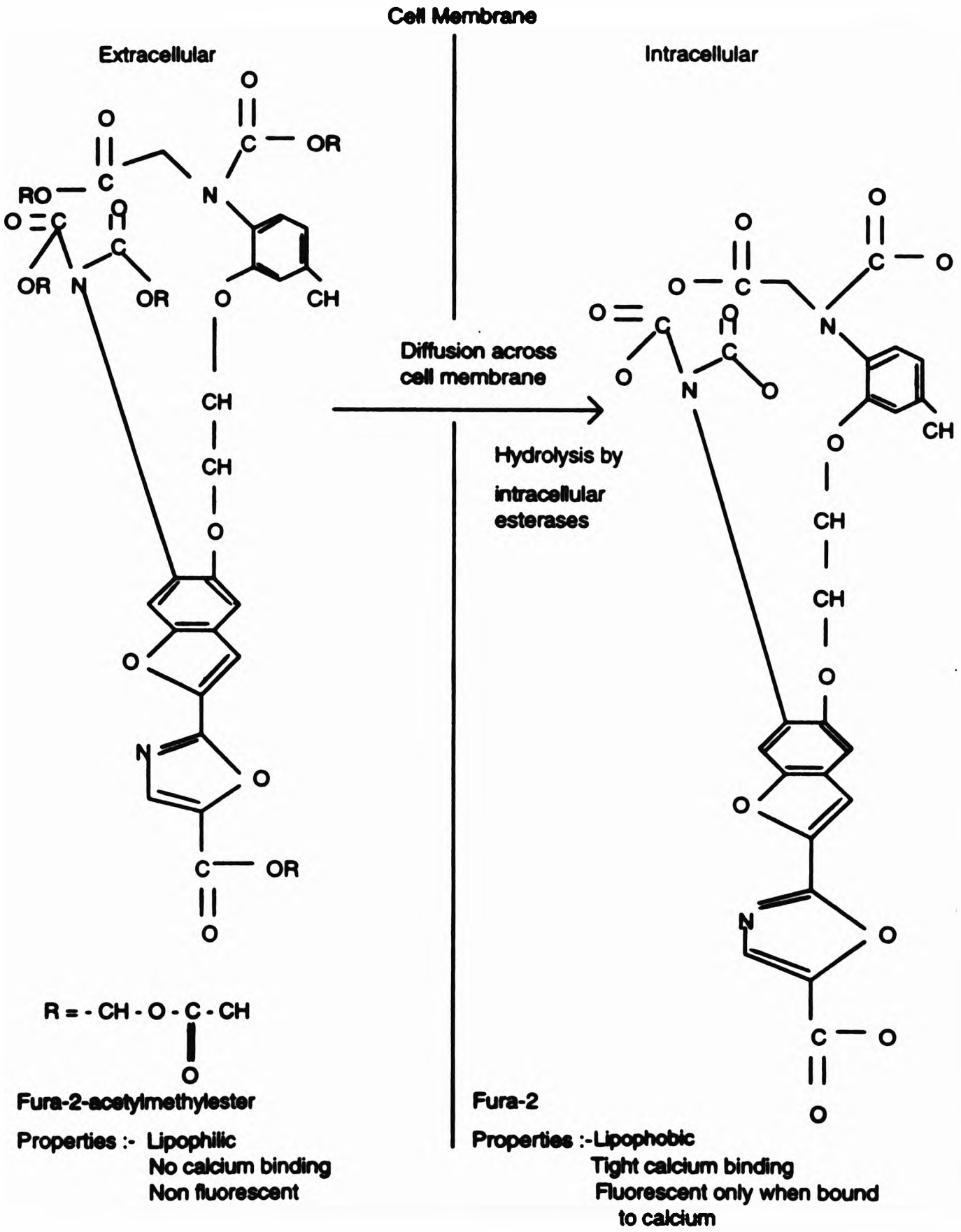


Figure 6.XXI. Structures of Fura-2-AM and Fura-2.

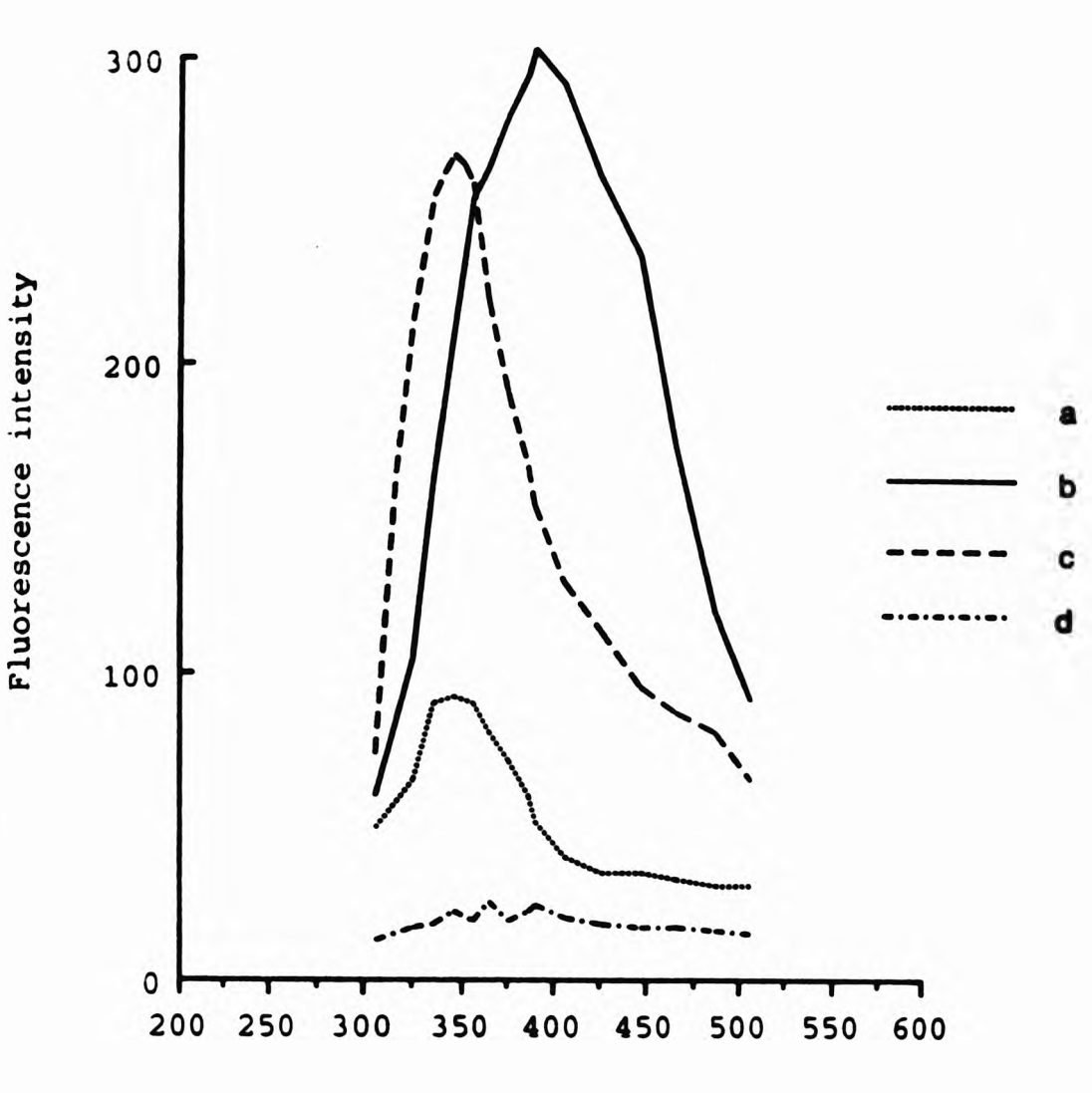
Figure 6.XXII. Photographs of spermatozoa loaded with Fura-2-AM.

↓ = Spermatozoa loaded with Fura-2-AM

6.5.4.5. Uptake and Hydrolysis of Fura-2.

Suspensions of *Fucus serratus* spermatozoa were incubated with Fura-2-AM (2 μM), for 3 hours and then extensively washed to free them of external probe. The fluorescence intensity of incubated spermatozoa was then measured over the excitation range 300 → 500 nm, with the emission wavelength set at 500 nm. The resultant spectra are shown in Figure 6.XXIII and these show that the excitation peak of spermatozoa loaded with Fura-2-AM was 340 nm, which is characteristic of the free acid hydrolysis product Fura-2 (Grynkiewicz, *et al.*, 1985) whereas, the excitation peak of the unhydrolysed acetyl methylester form of Fura-2 is 385 nm (Fig. 6.XXIII). Thus, it is apparent that *Fucus serratus* spermatozoa have succeeded in accumulating Fura-2-AM, de-esterifying it to Fura-2, and retaining Fura-2 inside the cell.

Figure 6.XXIII. Excitation spectra of spermatozoa incubated with Fura-2-AM.

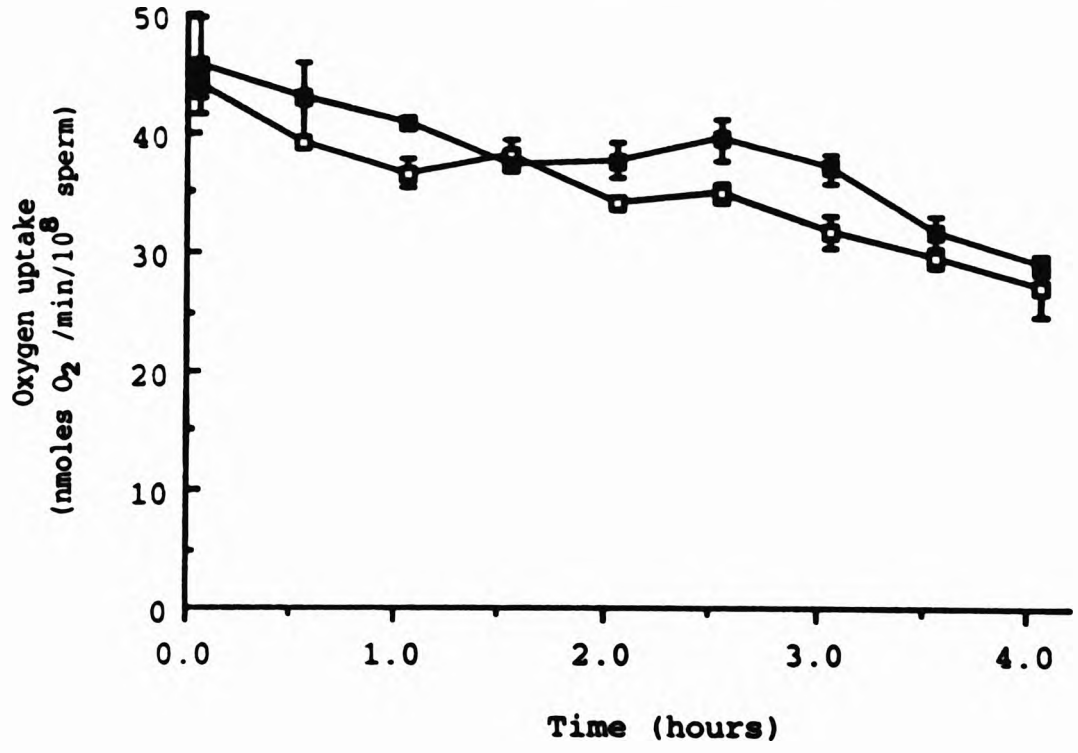


Excitation spectra (emission was set at 500 nm) for:
a = Fura-2 (2 μM);
b = Fura-2-AM (2 μM);
c = Spermatozoa from *Fucus serratus* loaded with Fura-2-AM (2 μM);
d = Autofluorescence of spermatozoa from *Fucus serratus*.
All the above scans were carried out in ASW. Spermatozoa were incubated with Fura-2-AM (2 μM) for 3 hours at 4°C, washed three times in ASW to remove extracellular dye and resuspended in ASW before scanning.

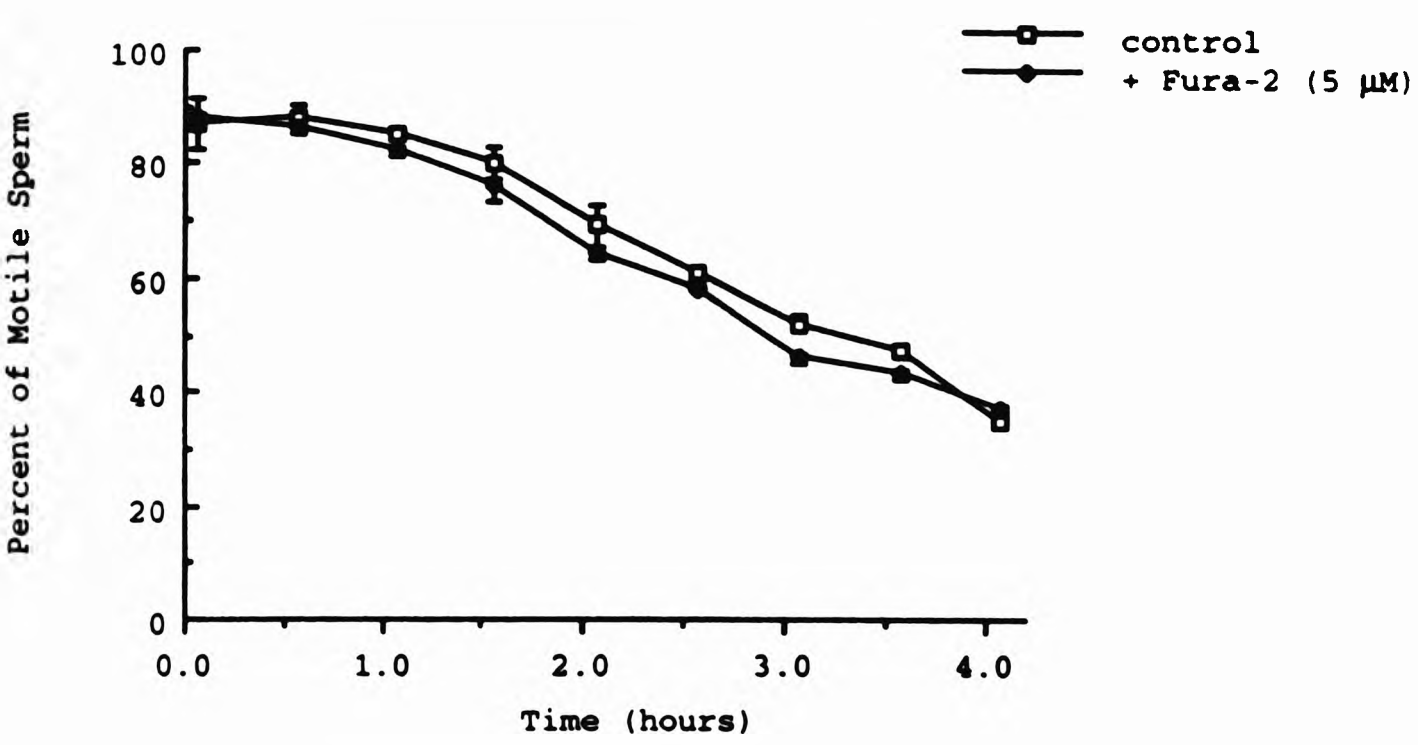
In order to determine whether Fura-2 had any toxic effects, suspensions of spermatozoa were loaded with Fura-2-AM (3 μM) and incubated for 4 hours. Samples were removed at regular intervals and assessed for both O_2 uptake and motility. The results in Figure 6.XXIV show that over a period of 3 hours O_2 uptake and the number of motile sperm decreased by 22% and 18% respectively compared with the 16% and 13% respective decreases observed in untreated controls. It would appear, therefore, that at this concentration Fura-2 has little toxic effect on spermatozoa of *Fucus serratus*.

Fig. 6.XXIII. The Effect of Fura-2-AM on the Oxygen Uptake and Motility of Spermatozoa.

A. Oxygen Uptake



B. Motility



Suspensions of spermatozoa loaded with or without Fura-2-AM (3 µM) were incubated for 4 hours at 10°C. Samples were removed at regular intervals and assessed for both O_2 uptake and motility as described in the Materials and Methods section. Values are means (n=6).

6.5.4.6. The Effect of Extracellular Ca^{2+} on the Concentration of Intracellular Ca^{2+}.

The intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) of *Fucus serratus* spermatozoa was measured as described in Materials and Methods and typical excitation spectra of spermatozoa loaded with Fura-2-AM are shown in Figure 6.XXV. The $[Ca^{2+}]i$ of these spermatozoa was calculated according to the equation of Grynkiewicz and colleagues (1985), and a detailed calculation can been seen in appendix 1. The $[Ca^{2+}]i$ of *Fucus serratus* spermatozoa incubated in ASW was 96.36 ± 1.20 nM (n = 6) however, when spermatozoa were suspended in OCaASW the $[Ca^{2+}]i$ decreased to 83.60 ± 1.55 nM (n = 5), Figure 6.XXVI shows typical spectra for spermatozoa incubated in OCaASW.

The effect of extracellular Ca^{2+} concentration on free intracellular Ca^{2+} concentration of spermatozoa is shown in Table 6.XII.

Table 6.XII. The effect of external Ca^{2+} on the $[Ca^{2+}]i$ as measured using the fluorescent probe Fura-2-AM.

Concentration of Extracellular Ca^{2+} mM	Concentration of Free Intracellular Ca^{2+} nM
0	83.60 ± 1.55
2	87.14 ± 2.00
5	89.30 ± 1.50
10	96.36 ± 1.20
15	98.88 ± 3.56
20	94.00 ± 2.86

Values are means ± SD (n = 3).

The results show that when the external concentration of Ca^{2+} was increased from 0 to 10 mM the intracellular free Ca^{2+} concentration of spermatozoa rose by 13%, indicating that external Ca^{2+} enters the cell probably through a Ca^{2+} channel. Attempts were made to measure $[Ca^{2+}]i$ in the presence of verapamil, an inhibitor of voltage-dependent calcium channels, but it unfortunately interfered with the fluorescence signal.

6.5.4.7. The Effect of External pH on the Concentration of Intracellular Ca^{2+}.

As the pH of the sea water into which *Fucus serratus* spermatozoa are released, plays an important part in the initiation of their motility and respiration, it seemed appropriate to examine whether the external pH had any affect on the concentration of intracellular free Ca^{2+} in these spermatozoa. The results are shown in Table 6.XIII.

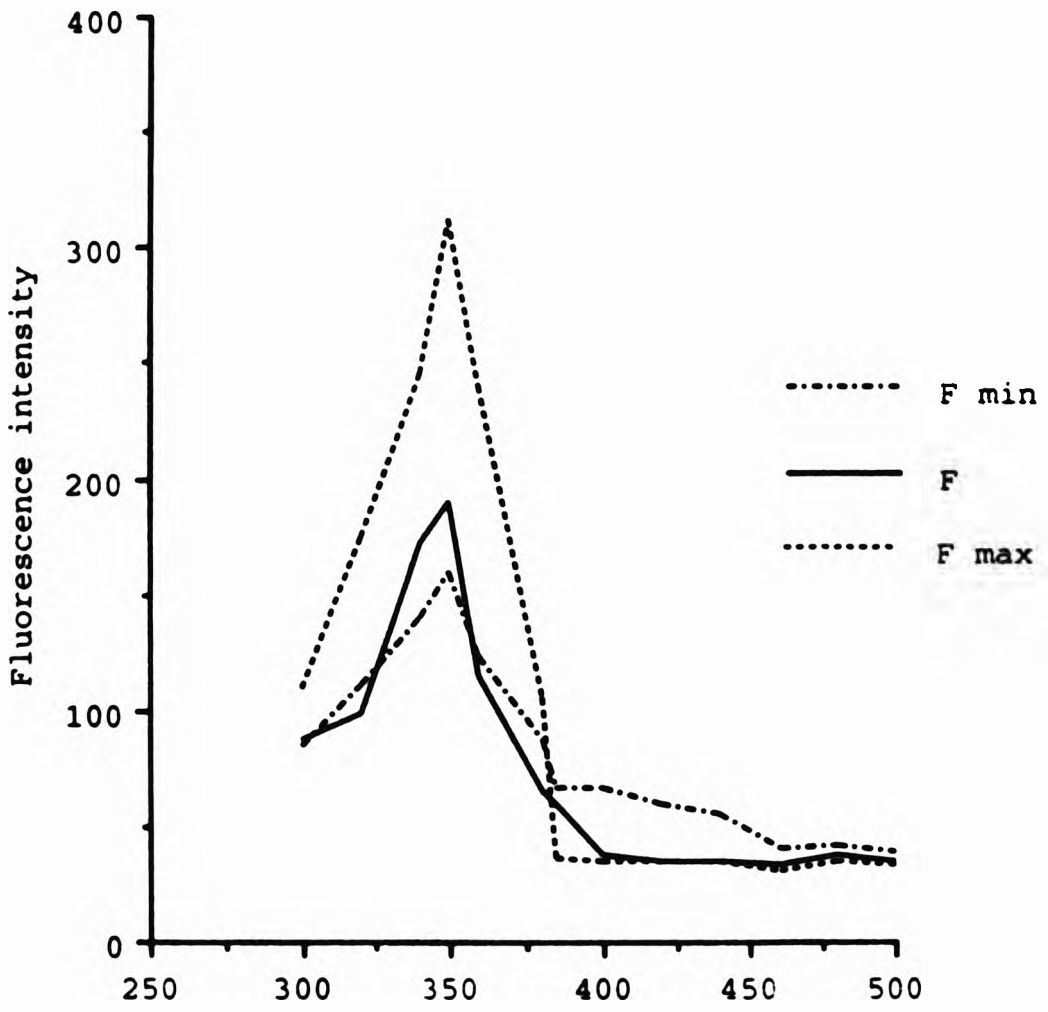
Table 6.XIII. The effect of external pH on the $[Ca^{2+}]i$ as measured using the fluorescent probe Fura-2-AM.

External pH	Concentration of Free Intracellular Ca^{2+} nM
6.0	63.00 ± 2.50
7.0	80.70 ± 1.88
8.0	95.50 ± 1.00
9.0	84.45 ± 2.10
10.0	79.15 ± 1.20

Values are means ± SD (n=3).

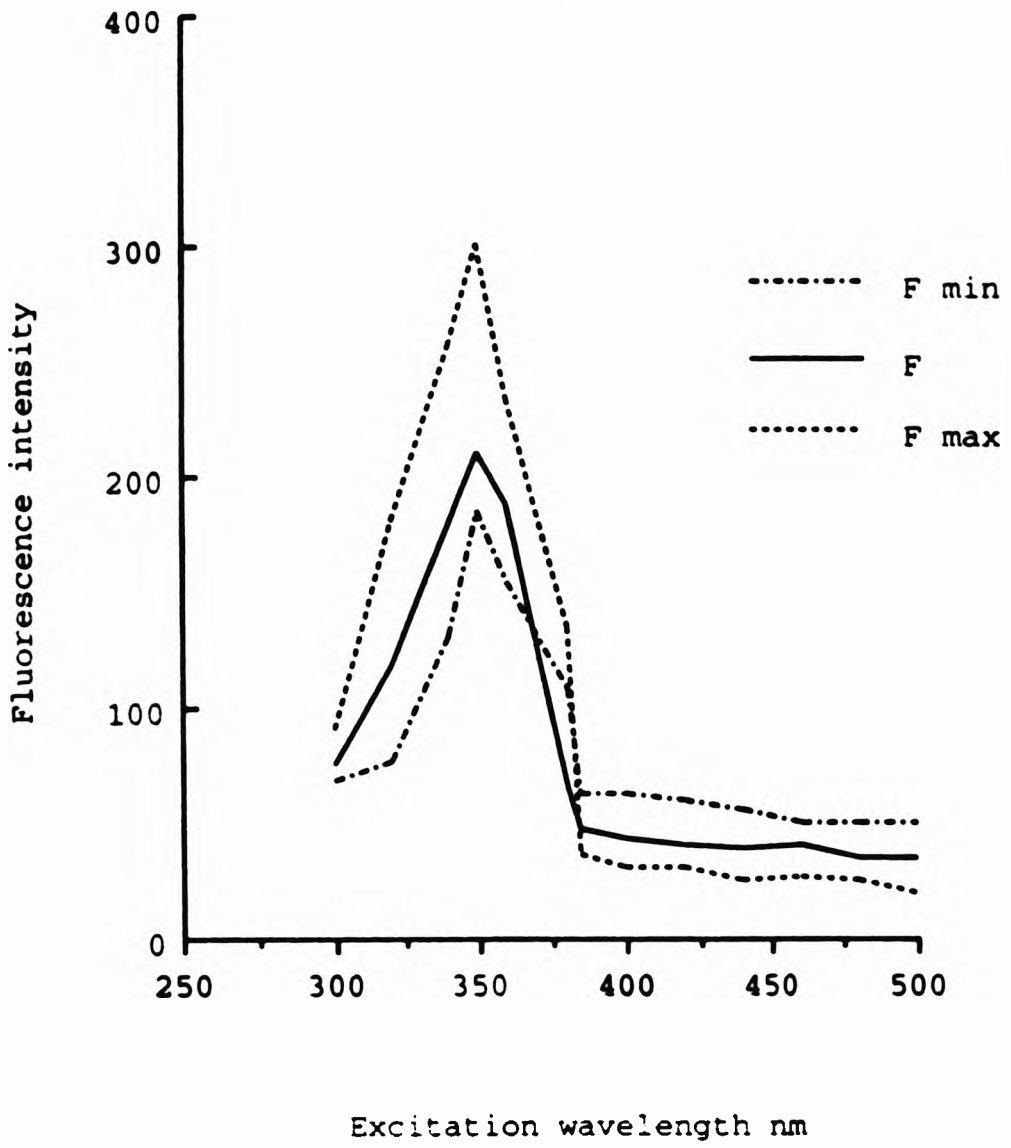
The results show that when the external pH of the ASW was acidic (pH 6.0), the $[Ca^{2+}]i$ of *Fucus serratus* spermatozoa was 34% lower than the $[Ca^{2+}]i$ of spermatozoa incubated in ASW at pH 8.0. Also, as the pH of the ASW was raised above 8.0, the $[Ca^{2+}]i$ decreased. Thus, it would appear that the pH of the sea water into which these spermatozoa are released affects the intracellular concentration of Ca^{2+}.

Figure 6.XXV. Typical Excitation Spectra for Spermatozoa suspended in ASW.



Typical excitation spectra (emission was set at 500 nm) for *Fucus serratus* sperm suspension incubated in ASW, in the presence of Fura-2-AM. The intracellular free Ca^{2+} concentration was calculated as described in the Materials and Methods section.
F_{min} = + EGTA + manganese
F = ASW
F_{max} = + Bromo A23187 + Ca^{2+}.

Figure 6.XXVI. Typical Excitation Spectra for Spermatozoa suspended in OCaASW.



Typical excitation spectra (emission was set at 500 nm) for *Fucus serratus* sperm suspension incubated in OCaASW. The intracellular free Ca^{2+} concentration was calculated as described in the Materials and Methods section.
F_{min} = + EGTA & manganese
F = OCaASW
F_{max} = + Bromo A23187 & Ca^{2+}.

6.5.4.8. The Effect of Monensin and Ouabain on the Concentration of Intracellular Calcium.

A rise in intracellular pH has been shown to play a role in the initiation of both motility and respiration in the spermatozoa of *Fucus serratus*. The increase in pHi is thought to be brought about through Na^+/H^+ countermovements and this study has shown that monensin, an activator of the Na^+/H^+ exchanger, increases the pHi in spermatozoa of *Fucus serratus* (see section 6.2.2.). The addition of monensin to sea urchin spermatozoa resulted in not only an elevation of pHi but an increase in $[Ca^{2+}]i$ as well (Harumi, *et al.*, 1992) These authors concluded that an elevation of pHi, due to Na^+/H^+ exchange through the plasma membrane may cause $[Ca^{2+}]i$ to increase.

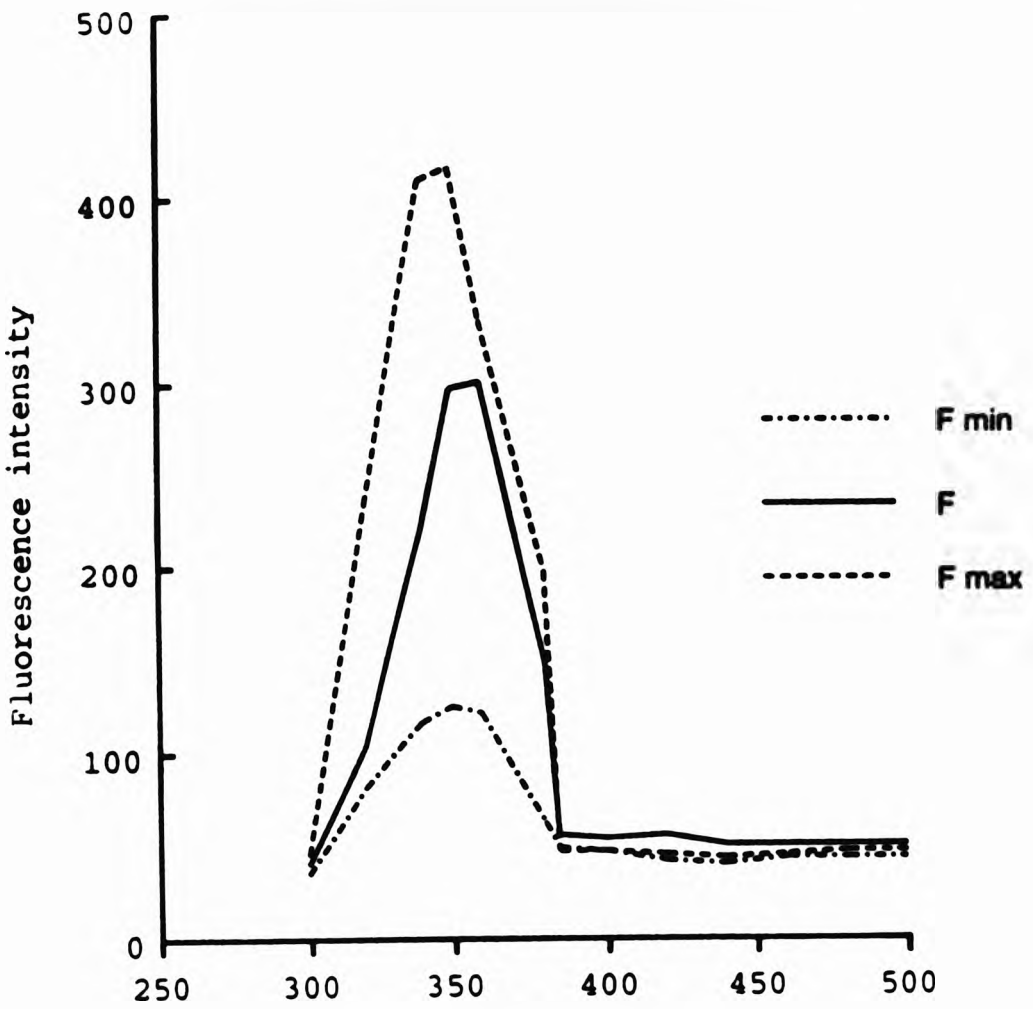
In order to examine whether monensin had any affect on the $[Ca^{2+}]i$ in spermatozoa of *Fucus serratus* suspensions of spermatozoa ($1x10^8$ sperm/cm^3) were incubated at 10°C for 15 minutes in the presence of monensin (0.1 µM). Spermatozoa, pelleted by centrifugation, were resuspended in 0.5 cm^3 OCaASW and loaded with Fura-2-AM as described in Materials and Methods. Figure 6.XXVII shows typical excitation spectra of spermatozoa incubated with monensin and loaded with Fura-2-AM and the free intracellular Ca^{2+} concentration was calculated using the equation described in Materials and Methods. The $[Ca^{2+}]i$ of spermatozoa stimulated with monensin was found to be 170 ± 6.75 nM (n=6), a 103% increase in $[Ca^{2+}]i$ when compared with spermatozoa incubated in OCaASW without monensin.

The Na^+/H^+ exchanger is driven by the transmembrane Na^+ gradient which in turn is generated by Na^+/K^+ ATPase. Thus, if a rise in pHi, brought about through Na^+/H^+ exchange, plays a role in stimulating $[Ca^{2+}]i$, then ouabain, an inhibitor of Na^+/K^+ ATPase, may cause a decrease in $[Ca^{2+}]i$. Suspensions of spermatozoa ($1x10^8$ sperm/cm^3) were incubated at 10°C for 15 minutes in the presence of ouabain (10 mM), after which time the suspensions were centrifuged, resuspended in 0.5 cm^3 OCaASW and loaded with Fura-2-AM, as described in Materials and Methods. Typical excitation spectra of spermatozoa incubated with ouabain are shown in Figure

6.XXVIII and the free intracellular Ca^{2+} concentration, calculated as described in Materials and Methods, was found to be 20.53 ± 4.70 nM (n=6). Therefore, the addition of ouabain to spermatozoa of *Fucus serratus* resulted in a 75% decrease in the $[Ca^{2+}]i$ when compared with spermatozoa incubated without ouabain.

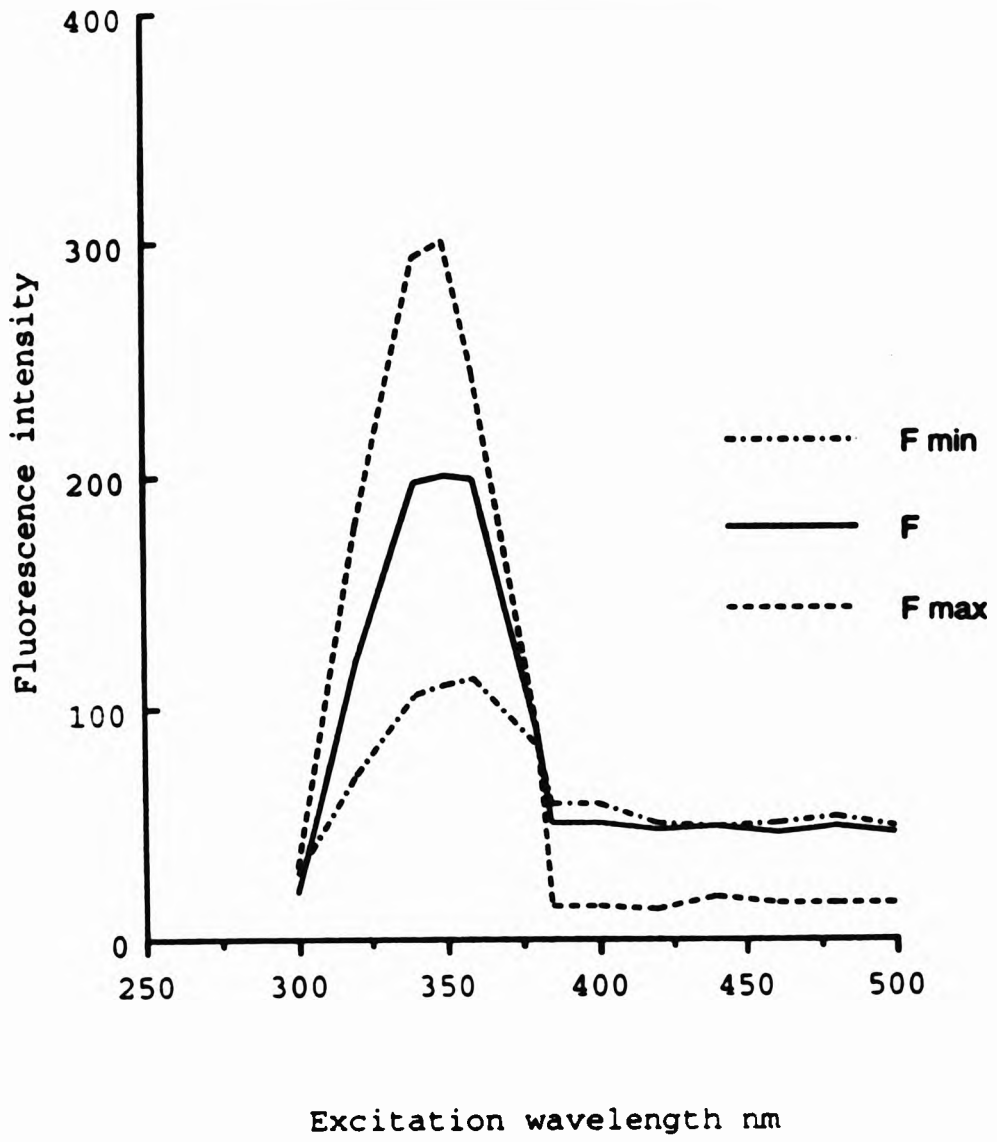
These results indicate that pHi, generated by Na^+/H^+ exchange which in turn is maintained via a Na^+/K^+ ATPase, causes the $[Ca^{2+}]i$ to rise in the spermatozoa of *Fucus serratus*.

Figure 6.XXVII. Typical Excitation Spectra for Spermatozoa suspended in OCaASW in the Presence of Monensin.



Typical excitation spectra (emission was set at 500 nm) for *Fucus serratus* sperm suspension incubated in OCaASW in the presence of Monensin (0.1 μM/cm^3). The intracellular free Ca^{2+} concentration was calculated as described in the Materials and Methods section.
F_{min} = + EGTA & manganese
F = + Monensin
F_{max} = + Bromo A23187 & Ca^{2+}.

Figure 6.XXVIII. Typical Excitation Spectra for Spermatozoa suspended in OCaASW in the presence of Ouabain.



Typical excitation spectra (emission was set at 500 nm) for *Fucus serratus* sperm suspension incubated in OCaASW in the presence of Ouabain (10 mM/cm^3). The intracellular free Ca^{2+} concentration was calculated as described in the Materials and Methods section.
F_{min} = + EGTA & manganese
F = + Ouabain
F_{max} = + Bromo A23187 & Ca^{2+}.

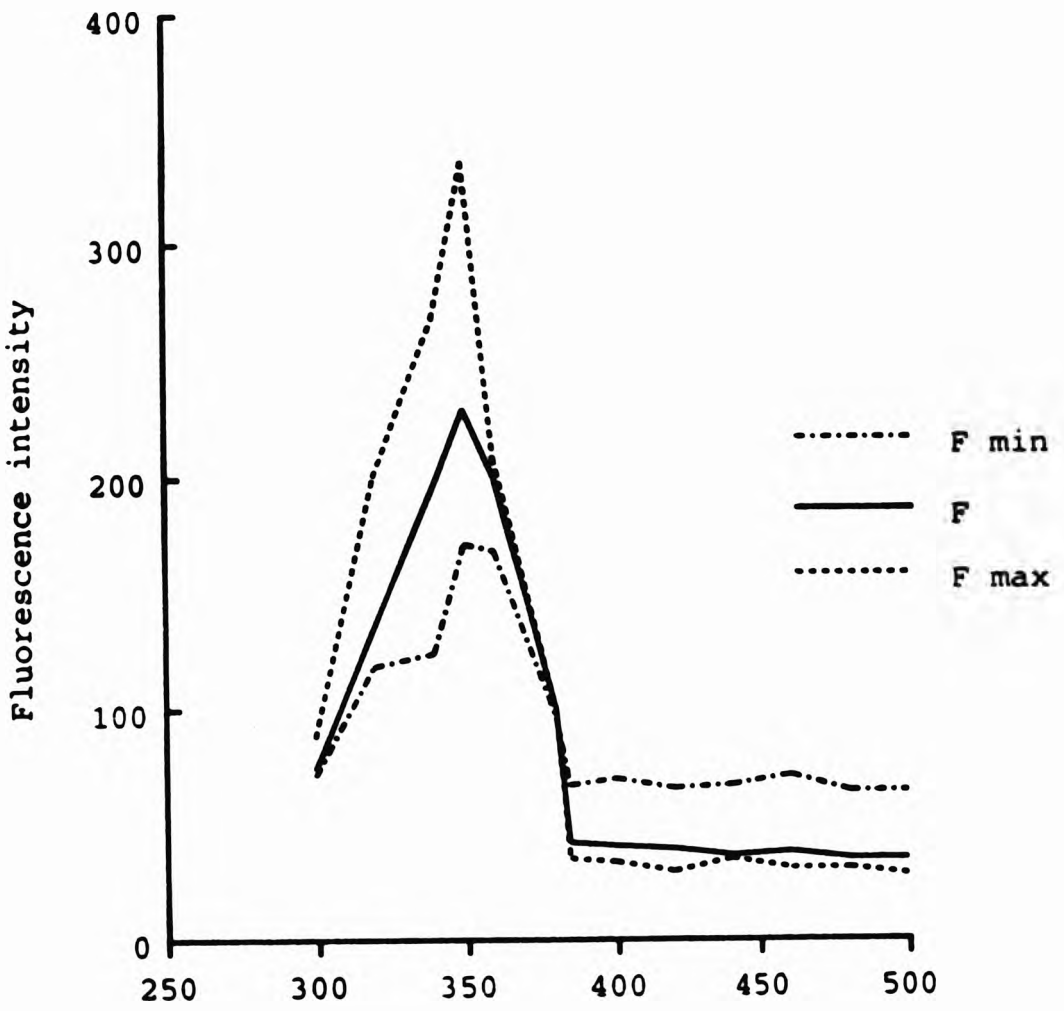
6.5.4.9. The Effect of PMA and H-7 on the Concentration of Intracellular Calcium.

The activator PMA and inhibitor H-7 of protein kinase C have been shown to stimulate and inhibit respectively both the O_2 uptake and motility of spermatozoa of *Fucus serratus*. Ca^{2+} has also been shown to be required for increased activity of PKC and, therefore, experiments were carried out to examine whether these agents affected the free intracellular Ca^{2+} concentrations of the spermatozoa. Suspensions of spermatozoa ($1x10^8$ sperm/cm^3) were incubated at 10°C for 15 minutes in the presence of either PMA (1 nM) or H-7 (20 µg/cm^3). Spermatozoa pelleted by centrifugation were then suspended in 0.5 cm^3 OCaASW and loaded with Fura-2-AM as described in Materials and Methods.

Typical excitation spectra of spermatozoa incubated with either PMA or H-7 and loaded with Fura-2-AM are shown in Figures 6.XXIX and 6.XXX respectively, and the free intracellular Ca^{2+} concentration was calculated using the equation described in Materials and Methods.

The $[Ca^{2+}]i$ of spermatozoa stimulated with PMA was found to be 156 ± 5.85 nM (n=5), and 30.8 ± 5.9 nM (n=6) when spermatozoa were inhibited with H-7. Spermatozoa suspended in OCaASW had an $[Ca^{2+}]i$ of 83.6 ± 1.55 nM (n=5), thus the presence of PMA stimulated the free intracellular Ca^{2+} concentration by 87%, whereas this was inhibited by 63% when spermatozoa were incubated with H-7.

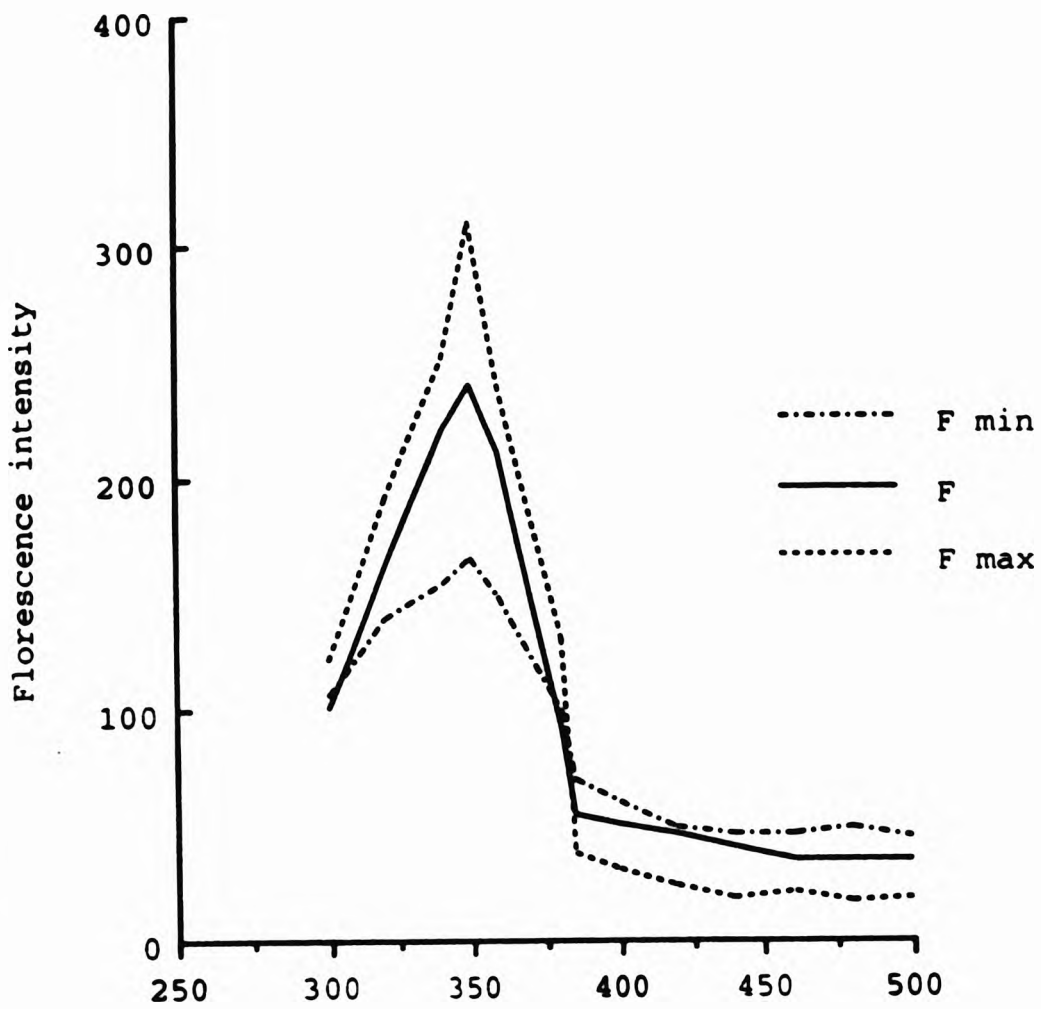
Figure 6.XXIX. Typical Excitation Spectra for Spermatozoa suspended in OCaASW in the Presence of PMA.



Typical excitation spectra (emission was set at 500 nm) for *Fucus serratus* sperm suspension incubated in OCaASW in the presence of PMA (1 nM). The intracellular free Ca^{2+} concentration was calculated as described in the Materials and Methods section.

F_{min} = + EGTA & manganese
F = + PMA
F_{max} = + Bromo A23187 & Ca^{2+}.

Figure 6.XXX. Typical Excitation Spectra for Spermatozoa suspended in OCaASW in the Presence of H-7.



Typical excitation spectra (emission was set at 500 nm) for *Fucus serratus* sperm suspension incubated in OCaASW in the presence of H-7 (20 μg/cm^3). The intracellular free Ca^{2+} concentration was calculated as described in the Materials and Methods section.
F_{min} = + EGTA & manganese
F = + H-7
F_{max}= = + Bromo A23187 & Ca^{2+}.

CHAPTER 7

Discussion

In these studies the mechanisms by which spermatozoa acquire their motility were investigated in sperm from both a mammalian source, viz. rat, and from the brown alga *Fucus serratus*. Experimental work was begun with the rat sperm and, later studies were carried out with sperm of *Fucus serratus*.

7.1. Mammalian Spermatozoa.

Mammalian spermatozoa acquire the capacity for motility during passage through the epididymis (Bedford, 1975). Spermatozoa isolated from the caput region of the epididymis are essentially immotile; 1-2% show a slight twitching motion, whereas the majority of sperm collected from the caudal region show vigorous progressive motion. Although the biochemical mechanisms by which this motility is acquired are not fully understood. There are three regulatory factors that either singularly or in combination seem to control the motility of mammalian spermatozoa. These are:

1) intracellular pH

2) cAMP

3) calcium.

7.1.1. Intracellular pH.

Intracellular pH (pHi) has been shown to regulate the initiation of mammalian sperm motility in a variety of species, including bovine (Babcock, *et al.*, 1983; Vijayaraghavan, *et al.*, 1985; Majumder, 1990) and sea urchin (Lee, *et al.*, 1983). Vijayaraghavan and colleagues (1985) have shown that bicarbonate induces an increase in intracellular pH in bovine spermatozoa and concomitant with an increase in motility bicarbonate has also been shown to contribute to the acquisition of motility

in sperm from salmonide fishes (Morisawa, *et al.*, 1993). As sperm pass from the testis to the sperm ducts the concentration of seminal bicarbonate increases (Morisaw, *et al.*, 1993).

In this study the effect of pH on the development of motility in rat spermatozoa was assessed. Sperm collected from caudal and caput epididymis were incubated in the presence of bicarbonate, at various concentrations within the physiological range of 1 to 50 mM. Such conditions resulted in induction of motility in caput epididymal spermatozoa and further increased the motility of caudal epididymal spermatozoa. Bicarbonate normally enters cells through a bicarbonate/chloride exchanger, which acts by exchanging external Na^+ and HCO_3^- for internal H^+ and Cl^-. This exchanger is inhibited by stilbene derivatives such as SITS and DIDS (L'Allemain, *et al.*, 1985) and the addition of SITS to rat caput and caudal epididymal spermatozoa substantially reduced the bicarbonate-induced stimulation of motility. These results suggest that bicarbonate plays a crucial role in the onset of motility and enters the cell via a bicarbonate/chloride exchanger. The entry of bicarbonate would result in extrusion of H^+ causing an increase in pHi, which may be a key factor in the initiation of motility. Bicarbonate has also been shown to activate adenylate cyclase, the enzyme which catalyses the formation of cAMP (Okamura, *et al.*, 1985; Rojas, *et al.*, 1992), and therefore, may act as the first physiological messenger for this enzyme. If this is the case then the activation of motility in rat spermatozoa may be due to a combination of an increase in pHi and in the intracellular levels of cAMP.

A Na^+/H^+ exchange system which is present in the plasma membrane of numerous animal cells has been shown to play a critical role in the regulation of pHi in sea urchin spermatozoa (Lee, *et al.*, 1984a & b). This enzyme mediates the counter-transport of Na^+ into the cell and H^+ out of the cell. In the present studies monensin, a monovalent cationic ionophore which catalyses Na^+/ H^+ exchange (Pressman, 1976; Hansborough and Garbers, 1981), caused an increase in the motility of rat

spermatozoa from both regions of the epididymis. However, the extent of stimulation of motility of the two epididymal spermatozoa populations differed with, once again, the greater degree of stimulation occurring in caput spermatozoa. Thus, it would appear that an increase in pHi has a greater effect on caput epididymal spermatozoa, than on the more mature caudal epididymal spermatozoa. This is not unexpected as the caput sperm are virtually immotile in the absence of stimulatory agents, whereas the caudal sperm already show some degree of motion. In a number of mammalian species including bovine, the pHi of caudal epididymal spermatozoa is more alkaline than that of caput epididymal spermatozoa (Majumder, 1990), and appears to be directly related to the extent of motility. The present studies strongly suggest that an increase in pHi is also required for the initiation of motility in rat spermatozoa.

7.1.2. Adenosine 3',5'-cyclic Monophosphate.

Cyclic nucleotides have been shown to play a role in the acquisition of the capacity for motility which occurs as mammalian spermatozoa travel through the epididymis (Hoskins, *et al.*, 1974; Hoskins and Casillas, 1975; Vijayaraghavan and Hoskins, 1990). Spermatozoa from the bull and ram exhibit an increase in cAMP levels during transit through the epididymis (Hoskins, *et al.*, 1974; Aman, *et al.*, 1982). On the other hand, cAMP levels in hamster spermatozoa have been shown to decrease during epididymal transit (Del Rio and Raisman, 1978; White and Aitken, 1989).

cAMP is synthesised through the activation of a membrane-bound enzyme, adenylate cyclase. Forskolin, a diterpene, is a known activator of adenylate cyclase (Seaman and Daley, 1981a & b; Vijayaraghavan, *et al.*, 1985) and when rat spermatozoa were incubated with this compound the motility of the more mature

caudal sperm was stimulated, but this compound had little effect on the motility of immature caput spermatozoa. If however, bicarbonate was present forskolin induced a marked stimulation in the motility of these immature spermatozoa. As already discussed quiescent spermatozoa, isolated from the caput region of the epididymis, have been reported to have an acidic pHi (Vijayaraghavan, *et al.*, 1985) and an elevation in pHi, induced through the entry of bicarbonate is required for induction of motility (Vijayaraghavan, *et al.*, 1985; Majumder, 1990). It may that forskolin can only stimulate adenylate cyclase and increase the intra-sperm levels of cAMP after this initial induction has occurred. In mature caudal epididymal spermatozoa the primary stimulus needed for the initiation of sperm motility is already present, and forskolin is able to stimulate cAMP production in the absence of external bicarbonate. Both bicarbonate and forskolin have been reported to activate adenylate cyclase in bovine caudal spermatozoa (Vijayaraghavan, *et al.*, 1985; Okamura, *et al.*, 1985; Rojas, *et al.*, 1992) and the findings of the present study indicate that these compounds in combination have a greater stimulatory effect on adenylate cyclase than they have separately.

The cellular levels of cAMP not only depend upon the rate of cAMP synthesis but also on its rate of degradation and a cAMP-specific phosphodiesterase catalyses the conversion of cAMP → 5'AMP. Theophylline, a phosphodiesterase inhibitor (Vijayaraghavan, *et al.*, 1985) induced a significant rise in the percent of motile spermatozoa in both epididymal populations of rat spermatozoa. However, once again a significantly greater enhancement of motility was observed when this compound was added to the caput epididymal spermatozoa in the presence of bicarbonate, but not in the case of the caudal epididymal spermatozoa. Further, in the presence of bicarbonate lower concentrations (1-2 mM) only were required to induce maximal motility in rat caput epididymal spermatozoa whereas in its absence higher concentrations of theophylline (20 mM) were needed to induce maximal motility,

indicating that bicarbonate increased the sensitivity of caput epididymal spermatozoa to the stimulatory action of theophylline. These results are compatible with a number of different mechanisms for initiation of sperm motility. It is probable that the entry of bicarbonate causes an increase in the pHi of spermatozoa which in turn stimulates quiescent spermatozoa. At this stage cAMP may also be involved in the stimulatory mechanisms and low concentrations of theophylline are sufficient to increase cAMP levels such that motility is enhanced. Bicarbonate, is known to elevate intra-sperm levels of cAMP through the activation of adenylate cyclase (Okamura, *et al.*, 1985; Rojas, *et al.*, 1992). It is possible that initiation of motility in caput epididymal spermatozoa by bicarbonate is due to such activation of the adenylate cyclase in the plasma membrane of rat spermatozoa. Such a mechanism would be consistent with the observation that only low concentrations of theophylline are required to increase the levels of cAMP to concentrations needed for this initiation of motility in immature spermatozoa. Theophylline, by itself, is capable of initiating motility in immature caput epididymal spermatozoa and this may be due to its ability to elevate the intracellular concentrations of cAMP. However, given other results using forskolin it is more likely that theophylline acts as a weak base (pka 8.7) thus increasing the pHi of these spermatozoa.

The concentration of cAMP has been reported to increase, in a number of mammalian species, as spermatozoa transcend the epididymis (Vijayaraghavan, *et al.*, 1985), thus the levels of cAMP may already be higher in rat spermatozoa in the caudal than in the caput region of the epididymis. In this situation, the addition of either bicarbonate or theophylline may be sufficient to enhance the levels of cAMP, through the activation of adenylate cyclase, and further stimulate the motility of caudal spermatozoa whereas, the caput epididymal spermatozoa require both bicarbonate and theophylline to elevate cAMP levels to those needed to initiate motility.

In this study, circumstantial evidence obtained using bicarbonate, forskolin and theophylline would suggest that a rise in pHi is an essential prerequisite for the initiation of the motility of rat spermatozoa, as they pass though the epididymis and the intra-sperm levels of cAMP play a role in this process. cAMP generally causes phosphorylation, through a cAMP-dependent protein kinase, of key regulatory proteins. For example, tubulin and the dynein heavy chain are two proteins reported to be phoshorylated and activated in a cAMP-dependent manner (Tash and Means, 1982; 1983). The existence of such a mechanism in rat spermatozoa requires further investigation.

7.1.3. Calcium.

The role of extracellular Ca^{2+} in regulating the motility of mammalian spermatozoa is unclear, as both stimulatory and inhibitory effects have been reported. For example, external Ca^{2+} has been shown to stimulate the motility of hamster spermatozoa (Morton, *et al.*, 1974), inhibit the motility of dog spermatozoa (Tash and Means, 1982a & b) and to have no effect on the spermatozoa of rabbit (Vijayaraghavan and Hoskins, 1990). The reasons for these species differences are unclear, as very little is known about the mechanisms regulating the entry of Ca^{2+} into the cell and its subsequent effect on motility. However, levels of intracellular Ca^{2+} have been shown to have a role in the regulation of mammalian sperm motility (Garbers and Kopf, 1980; 1983). One system that regulates the intracellular concentration of Ca^{2+} is a voltage-dependent Ca^{2+} channel in the plasma membrane of spermatozoa (Breitbart, *et al.*, 1990) and verapamil is an antagonist of this Ca^{2+} channel. In these studies the motility of rat spermatozoa from both regions of the epididymis decreased in the presence of this inhibitor implying that a voltage-

dependent Ca^{2+} channel is present. The consequent decrease in intracellular Ca^{2+} levels, brought about by verapamil somehow triggers a drop in the percent of motile spermatozoa.

Considering the results obtained with agents that cause an increase in levels of cAMP, it is of interest that intracellular Ca^{2+} can also cause enhancement of the concentration of intracellular cAMP (Rasmussen, 1970), which is coupled with an initiation of motility in both guinea pig and hamster spermatozoa (Garbers, *et al.*, 1982). It may be that a similar mechanism operates on the entry of Ca^{2+} into rat spermatozoa and this is different from the mechanism in bovine spermatozoa where the intracellular concentration of Ca^{2+} was higher in immotile caput spermatozoa than in the more motile caudal spermatozoa (Vijayaraghavan and Hoskins 1990). These authors suggested that the higher Ca^{2+} concentrations in caput epididymal spermatozoa impeded motility by regulating cAMP levels, through either stimulating a calmodulin-dependent cAMP phosphodiesterase or inhibiting a Ca^{2+}-sensitive adenylate cyclase. Thus, it is clear that further studies on the role Ca^{2+} plays in the regulation of motility of rat spermatozoa need to be carried out. These could include the direct measurement of the intracellular Ca^{2+} concentrations using fluorescent indicators for example, Fura-2, in both caput and caudal epididymal spermatozoa.

Ca^{2+} is also known to be an activator of Ca^{2+}-dependent protein kinase C (Rotem, *et al.*, 1990 a & b; DeJonge, *et al.*, 1991). Phorbol diesters, such as phorbol 12-myristate-13-acetate (PMA) are compounds that stimulate this protein kinase C (PKC) (Castagna, *et al.*, 1982; MacEwen, *et al.*, 1993) and in the present studies the addition of PMA to mature rat spermatozoa enhanced their motility both in the presence and absence of exogenous bicarbonate. Thus, it may be that another action of Ca^{2+} in the initiation of motility of rat spermatozoa is the activation of PKC. However, as well as activating PKC, phorbol diesters have been reported to increase the levels of cAMP in hamster spermatozoa (Visconti, *et al.*, 1990). These workers

also showed that bicarbonate stimulated the PMA-dependent cAMP accumulation and that this increase was abolished when inhibitors of the bicarbonate/chloride exchanger, for example SITS and DIDS, were present. From these results Visconti and colleagues (1990) concluded that the transport of bicarbonate across the plasma membrane of hamster spermatozoa, and the activity of PKC could be linked to the regulation of cAMP synthesis. In the present study SITS also inhibited the PMA-induced stimulation of motility, suggesting a similar interaction between the entry of bicarbonate, the activity of PKC and increasing cAMP levels in the regulation of rat sperm motility. On the other hand it may be, simply, that a rise in intracellular pH, brought about through the entry of bicarbonate, may be required to induce maximal activity of PKC.

The motility of quiescent caput epididymal spermatozoa, in the absence of exogenous bicarbonate, were not significantly affected by PMA whereas, in its presence, motility was stimulated. As reported before the pHi of caudal epididymal spermatozoa is higher than that of caput epididymal spermatozoa (Vijayaraghavan, *et al.*, 1985) and it is possible that an increase in the pHi of caput epididymal spermatozoa is needed before stimulation of PKC activity and consequent activation of motility. In caudal epididymal spermatozoa the PMA alone can stimulate motility and rises in pHi, brought about through bicarbonate uptake, stimulates the motility of caudal epididymal spermatozoa further.

From these results it seemed highly probable that PKC is present in rat spermatozoa and is involved in initiation of motility. To further establish the presence of PKC in rat spermatozoa and its role in the regulation of sperm motility, an inhibitor of PKC, H-7 (Rotem, *et al.*, 1990a) was added to spermatozoa in the presence of bicarbonate. Caput epididymal spermatozoa were totally immobile and the motility of caudal epididymal spermatozoa was significantly inhibited. The results with PMA suggesting that PKC is present and is a key component of the systems regulating rat

sperm motility and this conclusion is reinforced by these results with H-7. It is likely that PKC causes the phosphorylation and thereby activation/deactivation of key regulatory proteins but this is still to be determined.

In conclusion, from the various results of this study on rat spermatozoa it seems clear that, as in other mammalian spermatozoa, pH plays a major role in the initiation of motility and this may be linked with cAMP levels and the activation of PKC. Until the initial pH change in spermatozoa has occurred between the caput and caudal epididymal regions of the rat, second messengers are not effective in stimulating motility, but the evidence clearly suggests that they are involved in the stimulation of motility after such pH changes have occurred.

7.2. *Fucus serratus* Spermatozoa.

The spermatozoa of *Fucus serratus* differ from mammalian spermatozoa in that they are released into sea water prior to fertilisation and the motility of these sperm is initiated upon their release into sea water.

A number of external factors appear to be involved in this initiation of motility including temperature, pH and the composition of the sea water into which the spermatozoa are released. Present studies on the motility of the spermatozoa of *Fucus serratus* have shown these activities are maximal when the temperature of the sea water is 10°C and the pH of sea water into which they are released is 8.0. At lower pH values both motility and respiration are inhibited. These results might well be expected as the general temperature and pH of the seas where these spermatozoa are released is around 10°C and 8.0 respectively. In addition the sea water is obviously a critical component in sperm motility as spermatozoa diluted into sea water with reduced Na^+, Ca^{2+} and Mg^{2+} levels are considerably less motile than those released into normal ASW. This inhibition of motility can be reversed by the reintroduction of the appropriate ion.

7.2.1. Respiration of Spermatozoa from *Fucus serratus*.

Respiration of spermatozoa in this study was monitored using an O_2 electrode. As these sperm have a chloroplast which may produce O_2 care was taken not to carry out experiments in bright light. Further the electrode is surrounded by a water jacket which would absorb some light. Nevertheless it is possible that photosynthesis was occuring at a very low level. However, all treated sperm were compared with

untreated controls. Oxygen uptake was decreased by a number of agents and where it was elevated this was often by a considerable amount. Therefore, it seems unlikely that a very low photosynthetic rate would alter the conclusions drawn from the results of these experiments.

Movement of spermatozoa requires energy and this is provided by the hydrolysis of ATP, in particular, by dynein ATPase which is localised in the flagella. Spermatozoa are relatively simple cells and in those studied ATP is primarily formed by the respiration of mitochondria (Christen *et al.*, 1982) in which ADP is phosphorylated to ATP as electrons are transferred from NADH or $FADH_2$ to O_2 by a series of electron carriers. Respiration of the spermatozoa of *Fucus serratus* is sensitive to rotenone, antimycin A and potassium cyanide indicating that oxygen utilisation results from electron transport through the whole span of the mitochondrial electron transport chain similar to that found in other species. Further, oligomycin inhibits respiration suggesting that this electron transport is coupled to oxidative phosphorylation. The rate of respiration in dense suspensions of spermatozoa was lower than that in diluted suspensions. In diluted sperm suspensions the addition of DNP, an uncoupling agent of oxidative phosphorylation, only had a slight effect on the respiratory rate although it did reverse oligomycin inhibition. However, DNP did enhance the O_2 uptake of dense sperm suspensions. Thus, it could be concluded from these results that respiration coupled to oxidative phosphorylation occurs at a maximum rate in a diluted sperm suspension, whereas those in dense suspensions respire at a considerably reduced rate. Therefore, the mitochondria of spermatozoa in a dilute suspension are in state 3, as defined by Chance and Williams 1956, in which the availability of ADP does not limit the respiratory rate. Thus, in diluted suspensions, spermatozoa either control their ATP consumption to exactly match its synthesis, or they split ATP quicker than they phosphorylate ADP. Spermatozoa in a diluted suspensions swam vigorously where as, those in a dense suspensions were

largely immotile. The more motile sperm in dilute suspensions would have higher ADP levels resulting from ATP utilisation by the dynein ATPase which in turn would stimulate mitochondrial respiration (Brokaw and Benedict, 1968; Christen *et al.*, 1982, 1983). Spermatozoa in dense solutions probably compete for the available oxygen reducing respiratory rates, once again suggesting that these are closely linked to motility.

7.2.2. Intracellular Mechanisms associated with activation of motility.

7.2.2.1. Intracellular pH.

As described earlier internal pH is an important regulatory factor of motility of spermatozoa from a number of species including bovine, sea urchin (Vijayaraghavan, *et al.*, 1985; Roos and Boron, 1981; Schackman, *et al.*, 1978; 1981; Christen, *et al.*, 1982; 1983; Lee, 1984a &b) and rat (this thesis).

The pH of the sea water into which *Fucus serratus* spermatozoa are released greatly affects both their respiration and motility suggesting that pHi is also important in the initiation of motility in this species. The pH sensitive fluorescence probe, BCECF-AM, was used in this study to measure intracellular pH (pHi). BCECF-AM was taken up and fully hydrolysed to the free acid (BCECF) within three hours, as shown by a shift in the excitation spectra from a maximum at 465 nm, characteristic of BCECF-AM, to 490 nm, which is attributable to the free acid. No intermediate products were formed during this process and the hydrolysis of BCECF-AM had no adverse effect on either the O_2 uptake or motility of spermatozoa of *Fucus serratus*. Modifying the pH of the dilution medium is one experimental method which has been used to alter the intracellular pH of spermatozoa from a number of species such as sea urchin (Christen, *et al.*, 1982). Measurements of pHi in sperm from *Fucus serratus*

showed that as the extracellular pH of ASW was lowered from 8.0 to 5.0, the pHi decreased from 8.0 to 6.1. In these studies changes in the external pH, and by inference pHi, closely correlated with changes in sperm respiration and motility. It is of considerable interest that this mechanism operates in *Fucus* as although, pHi is known to influence motility in mammalian and some invertebrate sperm, the motility of other invertebrate sperm for example Horseshoe crab, changes independently of pHi (Clapper and Epel, 1981).

A number of mechanisms by which this change in pHi is achieved have been described for other species. Lee and co-workers (1982) have found that pHi for sea urchin spermatozoa in ONaASW is more acidic than when Na^+ is present. As the pHi of these spermatozoa was affected by both the pH and Na^+ concentration of the ASW it was proposed that regulation might involve Na^+-dependent H^+ movements (Lee, *et al.*, 1980; 1982). Similar proposals have also been made for changes in pHi in other cell types including sea urchin eggs (Johnson and Epel, 1976) and snail neurones (Meech and Thomas, 1980). As similar results with ONaASW were found with *Fucus serratus* spermatozoa, experiments were devised to identify such ion movements in this species. Monensin, a monovalent cationic ionophore, catalyses neutral Na^+/H^+ exchange across cell membranes (Pressman, 1976; Hansborough and Garbers, 1981). When added to spermatozoa from *Fucus serratus* the O_2 uptake was activated suggesting Na^+/H^+ exchange could be a key factor in motility of these sperm. The Na^+/H^+ exchanger is inhibited by amiloride in other cells (Johnson and Epel, 1976) and addition of this compound to spermatozoa of *Fucus serratus* resulted in an inhibition of both respiration and motility. The evidence clearly points to the presence of a Na^+/H^+ exchanger in the membranes of spermatozoa of *Fucus serratus*. In spermatozoa of sea urchin, the activation of such a Na^+/H^+ exchanger leads to the extrusion of H^+, producing an increase in intracellular pH (Lee, 1984a & b). In the present study intact spermatozoa suspended in Na^+-free ASW are virtually

immotile and elicit no decrease in the pH of the medium but on the addition of Na^+ a decrease in the pH of the medium by 0.4 pH units is observed. Intracellular pH, measured using BCECF-AM, was lower in spermatozoa suspended in ONaASW than in those suspended in ASW and the motility of these spermatozoa increased to over 80% when Na^+ was added. The intracellular pH of *Fucus serratus* spermatozoa also decreased in response to amiloride and increased in the presence of monensin and the motility of these spermatozoa paralleled these changes. Thus it seems, that in a similar way to that observed in sea urchin (Nishioka and Cross, 1978; Christen, *et al.*, 1982, 1983; Lee, *et al.*, 1982, 1983; Lee, 1984a & b), changes in intracellular pH, probably regulated by Na^+/H^+ counter-movements, play a critical role in the regulation of respiration and motility of spermatozoa of *Fucus serratus*.

Another ionic component of sea water which affects the motility of *Fucus serratus* spermatozoa is the concentration of K^+ ions. The Na^+-dependent H^+ release was particularly sensitive to the K^+ concentration of the sea water. There was no acid efflux and motility was inhibited when spermatozoa were suspended in ASW containing a high concentration of K^+ (160 mM compared with normal 10 mM) nor did spermatozoa suspended in ONaASW, containing a high concentration of K^+, activate acid efflux on addition of Na^+ (360 nm). However, partial acid release could be induced, under these inhibitory conditions, by the addition of monensin, the Na^+/H^+ exchange ionophore. In addition to this the motility of *Fucus serratus* spermatozoa was also stimulated under these conditions. The Na^+/H^+ exchanger in sea urchin spermatozoa is electroneutral with a voltage-sensitive gate. At high concentrations, K^+ depolarises the membrane potential of sea urchin spermatozoa (Schackerman, *et al.*, 1981; Lee, 1984a & b; 1985; Harumi, *et al.*, 1992). The depolarisation of the transmembrane potential closes the gate and inhibits the Na^+/H^+ exchanger in both its forward, (Na^+ in-H^+ out) and reverse, (Na^+ out-H^+ in) directions (Lee, 1984a & b; 1985). Thus, one possible explanation of the inhibitory effect of increased K^+ on Na^+-

dependent H^+ extrusion and motility of *Fucus serratus* spermatozoa, is that the cell membrane of the spermatozoa depolarises and inactivates the Na^+/H^+ exchange. As a result acid would be trapped inside the spermatozoa fixing the intracellular pH resulting in immobilisation of the spermatozoa.

The ways in which changes in internal pH trigger acquisition of motility is unclear. However, it is possible that the flagella of spermatozoa may be subject to direct control by pH changes. Isolated flagella of *Fucus serratus* spermatozoa, which still had their membranes intact, had characteristics of H^+ efflux similar to those of intact spermatozoa. For example, isolated flagella exhibited no acid release in ONaASW but caused a decrease in the pH of the medium by 0.35 units on addition of Na^+. However, the slow metabolic Na^+-dependent H^+ release seen in intact spermatozoa was not observed in isolated flagella presumably because no mitochondria are present. Care was taken to ensure that all preparations of flagella were virtually free of intact spermatozoa. In agreement with the results obtained with intact spermatozoa Na^+-dependent acid release was inhibited by high external K^+ concentrations and this inhibition was again partially reversed by the addition of monensin, suggesting that the primary site for activation of motility in response to changes in H^+ ion concentration is in the flagella themselves. As these are the primary agents of movement such a mechanism would be direct and rapid.

As a consequence of an internal alkalisation process in spermatozoa, removal of protons which are continuously produced by metabolism would also imply a continuous influx of sodium (Bibring, *et al.*, 1984). The consequent increase in the internal Na^+ concentration in turn would decrease the driving force for H^+ extrusion. To counteract this tendency an active mechanism for the extrusion of Na^+ should exist in these spermatozoa. It has been shown that Na^+/K^+-ATPase pumps located in the plasma membrane of sea urchin spermatozoa play a role in regulating pHi by recycling Na^+ (Gatti and Christen, 1985). When Li^+ was substituted for Na^+ in ASW

both respiration and motility of spermatozoa from *Fucus serratus* were reduced. Lithium is a poor substitute for Na^+ in the Na^+/K^+ exchange suggesting the presence of this pump in *Fucus serratus*. Further, ouabain, a specific inhibitor of Na^+/K^+ ATPase, also inhibited both respiration and motility when it was added to suspensions of spermatozoa. These results indicate that motility of spermatozoa from *Fucus serratus* is regulated by pHi which is influenced by internal Na^+ levels which in turn are controlled by a Na^+/K^+ ATPase.

The entry of bicarbonate into a cell is another way in which intracellular pH can be raised (Boron, *et al.*, 1979) and as already discussed, the presence of bicarbonate has been shown to cause an elevation in the pHi of mammalian spermatozoa (Vijayaraghavan, *et al.*, 1985). In these sperm, bicarbonate enters a cell through a bicarbonate/chloride exchanger which acts by exchanging external Na^+ and HCO_3^- for internal H^+ and Cl^-. The O_2 uptake and motility of spermatozoa of *Fucus serratus* was inhibited by the inhibitor of this exchanger, SITS. In several mammalian spermatozoa including bovine, mouse and human as well as in sea urchin spermatozoa (Okamura, *et al.*, 1985) in addition to elevating pHi, bicarbonate also appears to be involved in the activation of adenylate cyclase. Therefore the SITS-induced inhibition of motility in *Fucus serratus* spermatozoa may be due to either an acidification of the internal pH and/or through decreased activity of adenylate cyclase with a consequent decrease in cAMP levels which may also be associated with initiation of motility.

As previously mentioned one way that an increase in pHi could trigger the motility of *Fucus serratus* spermatozoa is through its effect on dynein ATPase. At low external pH the activity of dynein ATPase appeared to be inhibited to a greater extent than that of respiration, for example at pH 7.0 dynein ATPase activity fell by 84% when compared with the activity observed when the external pH was 8.0, whereas O_2 uptake only decreased by 34% when the external pH was changed from 8.0 to 7.0. It

may be that an acidic pHi initially inhibits the activity of the dynein ATPase and respiration of the mitochondria is subsequently inhibited by the resultant drop in ADP concentration. This would provide a mechanism where changes in pH directly influence flagella movement. However, both coupled and uncoupled (FCCP-treated) respiration are inhibited under conditions that lead to an acidic pHi thus, an acidic pHi may inhibit both respiration and motility. Christen and colleagues (1983) have found that at low intracellular pH, FCCP causes the depolarisation of the potential across the mitochondrial membrane in sea urchin spermatozoa and, under these conditions, mitochondria are fully uncoupled. Uncoupled respiration was, however, not achieved even under conditions of pH that totally inhibit dynein ATPase activity in spermatozoa of *Fucus serratus*, suggesting more complex interactions where pHi affect other intracellular messengers. Thus, the availability of ADP determined by dynein ATPase activity is probably not the only reason for changes in mitochondrial activity. In the presence of vanadate, an inhibitor of dynein ATPase, both the coupled and uncoupled respiration of *Fucus serratus* spermatozoa were inhibited, implying that O_2 uptake requires a functional dynein ATPase even in the presence of an uncoupler. However, in this study the investigations looking at the effect of pH on the ATPase activity of permeabilized spermatozoa were carried out at pH 8.0. At this external pH the intracellular pH is alkaline (8.0) and thus, the inhibitory effect on respiration seen at acidic pHi did not exist therefore, vanadate might inhibit mitochondrial activity directly.

In vitro dynein ATPase has an alkaline pH optimum and its activity increases sharply around pH 7.5 (Gibbons and Gibbons, 1972). Motility of the axoneme is activated over the narrow pH range of 7.3 to 7.8 in permeabilized sperm preparations (Goldstein, 1979). This is also the pH range in which respiration and motility of sea urchin sperm are increased *in vivo* (Christen, *et al.*, 1982) and is similar to the range

which results in an increase in these two parameters in spermatozoa of *Fucus serratus*.

In conclusion, internal pH is likely to play a role in controlling the rate of ATP hydrolysis by dynein ATPase and this ATPase activity is limiting for the respiration of tightly coupled mitochondria in a manner similar to that of spermatozoa from sea urchin.

7.2.2.2. Adenosine 3',5'-cyclic Monophosphate.

The role of cAMP, in the regulation of motility of mammalian spermatozoa is well established (Hoskins and Casillas, 1975; Tash and Means, 1982; Vijayaraghavan, *et al.*, 1985). In part, evidence for this has come through the use of stimulators of adenylate cyclase, and inhibitors of cAMP-specific phosphodiesterases. In this study of spermatozoa of *Fucus serratus*, similar approaches were applied to see if cAMP had any role in the initiation of the respiration and motility of these spermatozoa, upon their release into sea water.

Forskolin is known to be a potent activator of adenylate cyclase in somatic cells (Seamon and Daley, 1981a & b) and has been shown to stimulate the motility of bovine spermatozoa, presumably through the activation of adenylate cyclase (Vijayaraghavan, *et al.*, 1985). However, it has been reported that forskolin does not interact with the adenylate cyclase located in spermatozoa (Stengal and Hanoune, 1984). It has also recently been shown that in porcine spermatozoa forskolin stimulates Ca^{2+} uptake, through a verapamil-sensitive Ca^{2+} channel and enhances the intracellular concentration of free Ca^{2+} (Okamura, *et al.*, 1993). A rise in intracellular Ca^{2+} is known to be important in the regulation of flagella motility in many mammalian species of spermatozoa (Majumder, *et al.*, 1990; Ashizawa, *et al.*, 1992).

Thus, the increase in both motility and respiration seen when forskolin was added to suspensions of spermatozoa of *Fucus serratus*, may be due to its ability to stimulate the uptake of external Ca^{2+}, thereby increasing intracellular Ca^{2+} levels rather than its ability to increase cAMP levels through the activation of adenylate cyclase. Further studies on possible changes in protein phosphorylation patterns in response to cAMP and identification of forskolin activated Ca^{2+} channels are necessary to resolve this problem.

Two phosphodiesterase inhibitors, viz. caffeine and theophylline significantly stimulated the percent of motile *Fucus serratus* spermatozoa and their O_2 uptake and enabled these spermatozoa to maintain this elevated level of motion for a period of three hours. In the absence of inhibitors the percent of motile spermatozoa fell over the same period of time suggesting that a rise in the intracellular level of cAMP does play a role in the regulation of sperm motility and respiration. A more direct means of assaying the influence of cAMP on the motility and respiration of spermatozoa is to incubate spermatozoa with cAMP directly. Dibutyryl cAMP (dbcAMP) has been shown to be more effective as an experimental agent than cAMP, due to its resistance to hydrolysis and its greater lipid solubility (Garbers, *et al.*, 1971). The addition of dbcAMP to spermatozoa of *Fucus serratus*, incubated in ASW at pH 8.0, resulted in an increase in both motility and respiration. The results in this study also showed a striking change in the relationship between motility and pH when these spermatozoa were stimulated with dbcAMP. When spermatozoa were suspended in ASW at a low pH ($6 \rightarrow 7.5$), the addition of dbcAMP greatly enhanced their motility however, as the pH of the ASW was increased towards the optimum of pH 8.0, the extent of dbcAMP-induced stimulation decreased. At the pH of normal sea water the role cAMP plays in the regulation of motility is minimised and therefore, once again these results support the idea that the pH of the ASW is the most significant factor in the regulation of sperm motility.

In this study indirect evidence, using caffeine, theophylline and dbcAMP points to the presence of cAMP in the spermatozoa of *Fucus serratus* and its participation in the regulation of respiration and motility of these spermatozoa. Even though cAMP has been reported to be present in many plants, for example Soya bean (Blowers and Trewavas, 1989), its exact role remains unclear. In mammalian cells, cAMP initiates responses through the activation of cAMP-dependent protein kinases, which phosphorylate and thereby control the activities of key regulatory proteins. However, all attempts to locate cAMP-dependent protein kinases in plants have so far been unsuccessful (Blowers and Trewavas, 1989). The evidence gained in this study further supports the existence and possible regulatory role of cAMP in plants.

If cAMP does have a second messenger role in the regulation of sperm motility and respiration, then a first messenger would be required to trigger an increase in the intracellular levels of cAMP, presumably through activating adenylate cyclase. The eggs of sea urchin contain a sperm-activating peptide, which has many biological effects on the spermatozoa, such as stimulating their motility and respiration rate as well, as activating a rise in the intracellular levels of cAMP (Harumi, *et al.*, 1992). The eggs of *Fucus serratus* secrete a pheromone-like sperm chemo-attractant (Fucoserraten), which produces strong chemotactic responses by spermatozoa (Muller and Serferadis, 1977; Muller, 1979) and this chemo-attractant can be mimicked by a variety of hydrocarbons, such as n-hexane (Cook, *et al.*, 1951). In this study both the O_2 uptake and motility of spermatozoa of *Fucus serratus* were significantly stimulated when suspended in ASW containing n-hexane and the chemo-attractant secreted by the eggs of *Fucus serratus* may similarly affect spermatozoa. However, whether such agents trigger a rise in the concentration of cAMP, possibly through binding to the cell membrane and activating adenylate cyclase remains to be investigated.

7.2.2.3. The Inositol Phosphate Pathway.

The inositol phosphate pathway is a common mechanism for the transduction of extracellular signals across the plasma membranes of somatic cells (Berridge, 1987 a & b). The hydrolysis of phosphoinositol, by phosphoinositol-specific phospholipase C (PI-PLC), has been reported to be involved in the activation of both human (Atreja and Anand, 1985) and sea urchin (Takei, *et al.*, 1984) spermatozoa. The PI-PLC mediated hydrolysis of phosphoinositide generates inositol trisphosphate, which mobilises intracellular Ca^{2+}, and diacylglycerol, which in the presence of Ca^{2+} activates protein kinase C (Rotem, *et al.*, 1992). As already discussed, there is indirect evidence for the presence of the enzyme in spermatozoa from various species. For example, it has been reported to be present in mature spermatozoa of pig epididymis (Kimura, *et al.*, 1984), in human ejaculated spermatozoa, where it is thought to play a role in flagella motility (Rotem, *et al.*, 1990a & b; De Jonge, *et al.*, 1991), and in this project in rat spermatozoa.

Fucus serratus on the other hand is a plant and although PKC has been reported to be present in plants (Anderson, 1989; Drobak, 1993)), its role remains unclear and therefore, it was of considerable interest to investigate whether PKC is involved in the activation of *Fucus serratus* spermatozoa. When activators and inhibitors of mammalian PKC were added to suspensions of these spermatozoa respiration and motility were enhanced and inhibited respectively. The presence of the phorbol diesters phorbol 12-myristate 13 acetate (PMA) and 4 β-phorbol 12,13 didecanoate (β-PDD), which are known activators of mammalian PKC caused activation of sperm. Phorbol diesters are not readily metabolised in somatic cells and therefore, only very low concentrations of these diesters are necessary to stimulate PKC and elicit a cellular response (Castagna, *et al.*, 1982). The concentrations used in this study were similar to those which elicit responses in somatic cells (Castagna, *et*

al., 1982), suggesting that PKC may be involved in the activation of spermatozoa of *Fucus serratus*. These results are surprising and exciting as the PKC isolated from plants so far, are not activated by phorbol esters and as yet no physiological activity has been connected to the addition of extracellular phorbol esters to higher plant cells (Anderson, 1989). Thus, the results in this study are the first to demonstrate a phorbol ester activated PKC in plants and show a physiological response to the addition of external phorbol esters to plant tissues. There is considerable evidence that PKC is dependent upon Ca^{2+} for activity however, in these studies PMA slightly enhanced motility and O_2 uptake in the absence of Ca^{2+} and, indeed, in the presence of EGTA, which was surprising. These results are not without precedent in that PKC mediated reactions elicited by another phorbol diester 12-O-tetradecanoyl phorbol-13-acetate (TPA) have been shown to be Ca^{2+}-independent in human platelets (Rink, 1987). Therefore, Ca^{2+}-independent activation of PKC cannot be ruled out in the activation/regulation of motility and respiration in spermatozoa. It is also possible that other mechanisms which are activated by PMA maybe involved in the activation of sperm motility, as this compound has been shown to stimulate the intracellular concentration of cAMP in hamster spermatozoa (Visconti, e*t al.*, 1990).

H-7, an inhibitor of PKC (Rotem, *et al.*, 1990a) markedly decreased both O_2 uptake and motility of *Fucus serratus* spermatozoa. However, PKC is not the only target for H-7 inhibition, which also inhibits cAMP-dependent protein kinase (Ki = 3.0 μ mol/l), (Hidaka, *et al.*, 1984). On the other hand, H-7 inhibited O_2 uptake and motility of spermatozoa even in the presence of an activator of PKC, implying that PKC is the main target of H-7 inhibition in these spermatozoa.

The results obtained, in this study, using phorbol diesters and H-7 imply that protein kinase C has a role in regulating motility and respiration in the spermatozoa of *Fucus serratus*, possibly through the phosphorylation of key regulatory proteins. One of the actions of PKC in animal cells is catalysing the phosphorylation and thereby

activation of the plasma membrane Na^+/H^+ exchanger, thereby inducing cytosolic alkalisation (Siffert and Akkerman, 1988; Tse, *et al.*, 1993). The rise in intracellular pH, which has been shown to be involved in the activation of *Fucus serratus* spermatozoa, is induced through Na^+/H^+ exchange, and if this exchanger is the target for PKC activity in these spermatozoa, it would be activated with the consequent effect on motility and respiration of these spermatozoa.

7.2.2.4. Calcium.

The role of external calcium in the initiation and/or regulation of sperm motility appears to vary in different species, as Ca^{2+} can have either a stimulatory or an inhibitory effect (Feng, *et al.*, 1988; Ashizawa, *et al.*, 1992). In plants, there is evidence to suggest that Ca^{2+} acts as a second messenger, in many physiological processes; such as, cell elongation and enzyme activation (Hepler and Wayne, 1985; Marme, 1989) but, the mechanisms by which Ca^{2+} regulates these processes are unresolved. A Ca^{2+}-dependent protein kinase activity has been reported to occur in the membranes of pea shoots (Hetherington and Trewavas, 1982) and a Ca^{2+}/calmodulin-dependent protein kinase activity has been demonstrated in the membranes of courgettes (Marme, 1989). As well as these two types of protein kinases, a Ca^{2+} and phospholipid-dependent protein kinase has been identified in the membranes of courgettes (Schafer, *et al.*, 1985). A link may also exist between the Ca^{2+} messenger system and protein kinase C. Although these three Ca^{2+}-dependent protein kinases have been located in plants, nothing is known about the biochemical or physiological function of the substrates, if any, which these kinases phosphorylate.

The results in this study show that Ca^{2+} plays an important role in initiating the motility and respiration of spermatozoa isolated from the brown alga, *Fucus serratus*.

In the presence of Ca^{2+}-free sea water (OCaASW) there were 70% fewer motile spermatozoa and the O_2 uptake of these spermatozoa decreased by 67%, when compared with spermatozoa incubated in normal ASW. However, upon the re-introduction of Ca^{2+} (10 mM) both the motility and O_2 uptake of these spermatozoa increased. The presence of a Ca^{2+} channel was also indicated in the spermatozoa of *Fucus serratus*, as the addition of verapamil inhibited both motility and respiration. These results show that spermatozoa of *Fucus serratus* behave in a similar manner to sea urchin sperm which display a marked stimulation of motility in external calcium concentrations up to 9 mM (Tash and Means, 1983; 1987), as might be expected given that they are both released into the same environment.

The intracellular concentration of Ca^{2+}, ($[Ca^{2+}]i$), has been shown to have an important role in controlling motility in mammalian spermatozoa (Garbers and Kopf, 1980; Ashizawa, *et al.*, 1992). Although the mechanisms regulating $[Ca^{2+}]i$ have not been completely elucidated, optimal Ca^{2+} concentration probably depends on the balance between Ca^{2+} uptake and release, which are controlled by Ca^{2+} pumps and channels in the plasma and mitochondrial membranes.

In this study, the intracellular Ca^{2+} concentration of *Fucus serratus* spermatozoa was manipulated with EGTA and the ionophore A23187. Unbound Ca^{2+} in a cell can be decreased to nanomolar concentrations by introducing a Ca^{2+}-specific chelator, such as EGTA and the addition of this compound to suspensions of *Fucus serratus* spermatozoa resulted in the inhibition of both O_2 uptake and motility. Non selective, non-localised Ca^{2+} entry across membranes is promoted by A23187, an ionophore (Reed and Lardy, 1972), which has been shown to be a useful tool for the study of Ca^{2+} flux in mammalian spermatozoa (Simpson, *et al.*, 1987). The presence of A23187 promoted an increase in the motility and O_2 uptake of *Fucus serratus* spermatozoa suspended in either ASW or ONaASW, but had no significant effect when spermatozoa were incubated in OCaASW containing EGTA. A23187 may

override both mitochondrial and plasma membrane pumps allowing free entry of Ca^{2+} and consequently an increase in $[Ca^{2+}]i$ (Simpson, *et al.*, 1986), implying that an increase in $[Ca^{2+}]i$ is a critical factor in the regulation of respiration and motility. This is in agreement with results from studies in many mammalian species where intracellular Ca^{2+} has been shown to be involved in sperm motility (Majumder, *et al.*, 1990; Ashizawa, *et al.*, 1992).

The direct measurement of $[Ca^{2+}]i$ has been carried out using fluorescent Ca^{2+} indicators Quin-2 and Fura-2 (Tsien, 1981; Tsein, *et al.*, 1982; Grynkiewicz, *et al.*, 1985) in numerous mammalian spermatozoa (Aitken, *et al.*, 1986; Babcock and Pfeiffer, 1987; Thomas and Meizel, 1988; Vijayaraghavan and Hoskins, 1989; 1990) as well as in zygotes of *Fucus serratus* (Brownlee, 1989). In this study the $[Ca^{2+}]i$ of spermatozoa of *Fucus serratus* was measured using Fura-2. The membrane-permeate acetoxymethylester form of Fura-2 (Fura-2-AM) showed a shift in the excitation spectrum, from that of Fura-2-AM to that of the free acid, Fura-2, after three hours in spermatozoa that had been washed three times. These results show that Fura-2-AM could be readily loaded into *Fucus serratus* spermatozoa and was subsequently intracellularly hydrolysed to the free acid. Further O_2 uptake and motility measurements of spermatozoa loaded with Fura-2-AM showed that, at the concentration of Fura-2 used and over the time period studied no toxic effects occurred. It therefore seemed that Fura-2 was a suitable indicator for measurements of intracellular free Ca^{2+} concentration of *Fucus serratus* spermatozoa.

As the extracellular concentration of Ca^{2+} was increased from 0 → 10 mM, the $[Ca^{2+}]i$ of *Fucus serratus* spermatozoa increased by 13%, This Ca^{2+} entry into the spermatozoa is most probably through a calcium channel as verapamil, a Ca^{2+}-channel blocker, prevented such increases. Unfortunately, verapamil interfered with the fluorescence signal and it was not possible to verify whether the uptake of

external Ca^{2+} into *Fucus serratus* spermatozoa occurred through a verapamil-sensitive channel, using this particular approach.

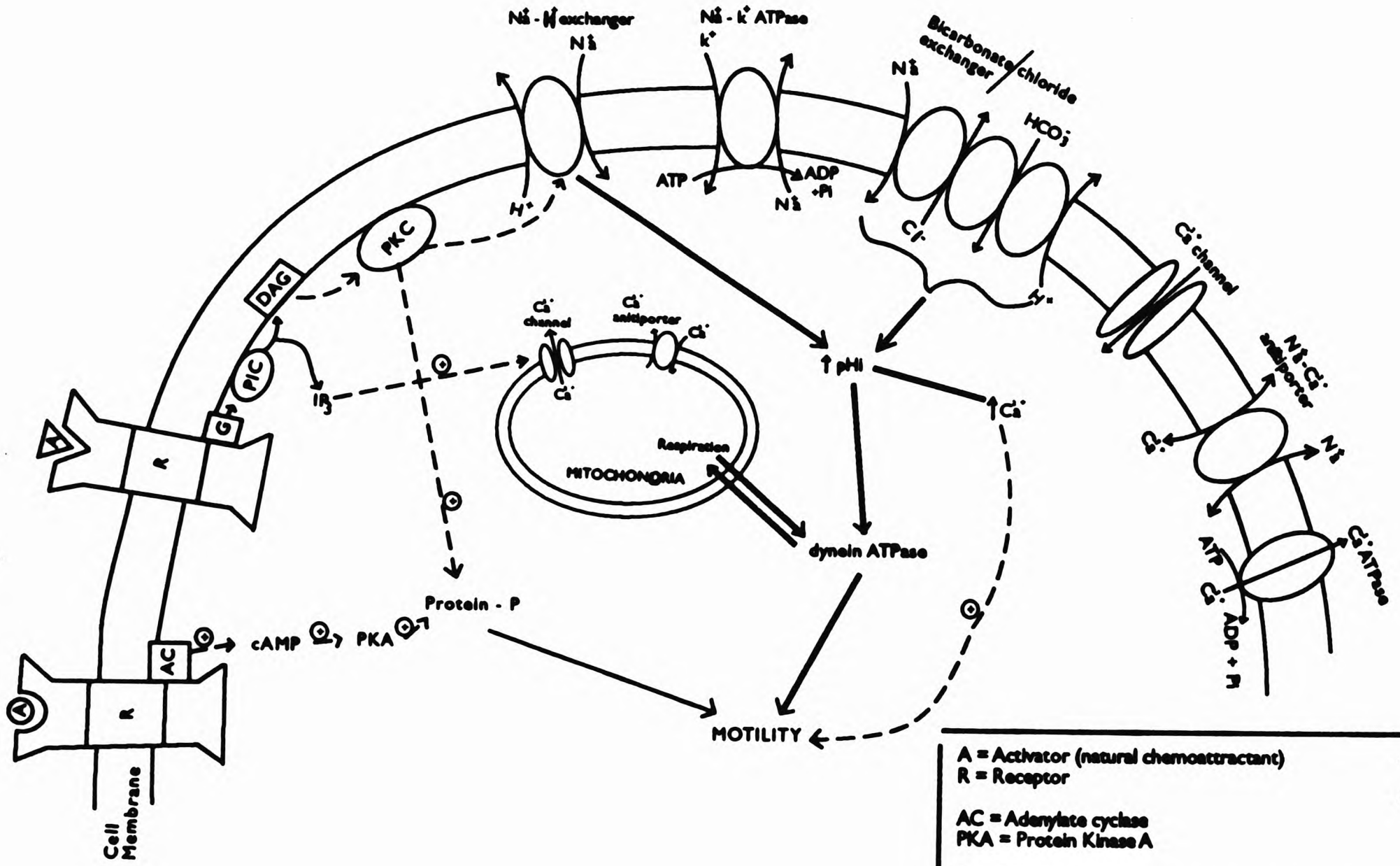
External pH also effects the $[Ca^{2+}]i$ of *Fucus serratus* spermatozoa and as already discussed the pH of the sea water into which spermatozoa are released is a critical factor in the regulation of their motility and respiration. Spermatozoa released into acidic ASW had an $[Ca^{2+}]i$ 34% lower than that of spermatozoa released into ASW at pH 8.0, which was the average pH of the sea water at the location where the plants were collected. The $[Ca^{2+}]i$ of these spermatozoa also decreased as the pH of the ASW rose above 8.0.

Results obtained in this study have also shown that modifying the pH of ASW is one way of affecting the intracellular pH of spermatozoa and a rise in pHi stimulates motility and respiration in *Fucus serratus* spermatozoa. This is in agreement with results obtained for other species, including sea urchin spermatozoa (Christen, *et al.*, 1982; 1983), where the increase in pHi is thought to be brought about through Na^+/H^+ contermovements. An increase in the pHi of *Fucus serratus* spermatozoa accompanied by activation of motility and respiration was seen in the presence of monensin, an activator of the Na^+/H^+ exchanger. However, monensin, not only increased the pHi of *Fucus serratus* spermatozoa, but also resulted in a 103% increase in the $[Ca^{2+}]i$ levels compared with control spermatozoa incubated in the absence of monensin. Monensin has also been shown to stimulate $^{45}Ca^{2+}$ uptake in mouse spermatozoa (Fraser, 1993). The Na^+/H^+ exchanger is driven by a transmembrane Na^+ gradient, generated by a Na^+/K^+ ATPase and ouabain, an inhibitor of this enzyme, induced a decrease in the $[Ca^{2+}]i$ of *Fucus serratus* spermatozoa. Fraser (1993) has suggested that monensin, through activating Na^+/H^+ exchange causes a rise in the intracellular concentration of Na^+ which sets in motion a sequence of ionic changes, including the depolarisation of the cell membrane and the influx of Ca^{2+} via voltage-dependent calcium channels. Thus, the entry of Na^+,

through Na^+/H^+ exchange, could activate the motility either by activating the entry of Ca^{2+} via voltage-dependent calcium channels and/or through increasing the pHi of these spermatozoa. An increase in pHi, as well as activating spermatozoa in its own right has also been shown to activate calcium channels in both invertebrate (Garcia-Soto, *et al.*, 1985; 1987) and mammalian (Babcock, 1988) sperm cells. The evidence from this current work indicates that spermatozoa from *Fucus serratus* may employ similar mechanisms for activation of motility and O_2 uptake, but more direct studies on Ca^{2+} channels need to be carried out before detailed pathways can be proposed.

The results in this study indicate that an increase in pHi by Na^+/H^+ exchange through the cell membrane, which in turn is maintained via a Na^+/K^+ ATPase, may cause an increase in the $[Ca^{2+}]i$ of *Fucus serratus* spermatozoa.

Protein kinase C activity may be involved in activating the motility and respiration of *Fucus serratus* spermatozoa and previous workers have shown that this enzyme activates/regulates cellular processes through catalysing the phosphorylation of key regulatory proteins, including in some cells the Na^+/H^+ exchanger (Siffert and Akkerman, 1988; Tse *et al.*, 1993). When the $[Ca^{2+}]i$ of *Fucus serratus* spermatozoa was measured in the presence of either the phorbol diester, PMA, or H-7 the $[Ca^{2+}]i$ was stimulated and inhibited by 88% and 37% respectively. It would seem, therefore, that PKC has a role in regulating the intracellular concentration of free Ca^{2+}. One possible mechanism consistent with these findings is that a chemo-attractant, released from *Fucus* eggs, activates PKC, which catalyses phosphorylation and thereby activation of the Na^+/H^+ exchanger of the cell membrane. The activation of this exchanger would in turn produce a rise in the intracellular pH of *Fucus serratus* spermatozoa, which increases the $[Ca^{2+}]i$, both of which stimulate motility and respiration by activating voltage-dependent Ca^{2+} channels.



A = Activator (natural chemoattractant)
R = Receptor

AC = Adenylate cyclase
PKA = Protein Kinase A

G = G protein
PIC = Phosphoinositol - specific phospholipase C
DAG = Diacylglycerol
IP_3 = Inositol 1,4,5 triphosphate
PKC = Protein Kinase C

Protein - P = Phosphorylated proteins
pHi = Intracellular pH
Ca^{2+} = Intracellular Ca^{2+}

→ = Proposed Pathway

⟶ = Elucidated Pathway

? = Possible mechanism fo
Ca^{2+} extrusion

Fig 7.1 Schematic representation of factors affecting the motility of Spermatozoa from *Fucus Serratus*

Many mechanisms seem to be involved in the regulation of the motility and respiration of *Fucus serratus* spermatozoa and the factors affecting the motility of these spermatozoa are shown in Figure 7.I.

The motility and respiration of *Fucus serratus* spermatozoa appears to be activated upon their release into sea water and the concentration of at least three ions in sea water viz. Na^+, Ca^{2+} and Mg^{2+} appear to be essential in this activation. It is evident that the most important of these is Na^+. The results of these studies suggest that the concentration of Na^+ is regulated via various channels and pumps, i.e. the Na^+/H^+ exchanger, the Na^+-dependent bicarbonate/chloride exchanger and the Na^+/K^+ pump. The presence of these exchangers/pumps in spermatozoa from *Fucus serratus* has been implicated by the use of specific inhibitors and activators. By mechanisms already discussed it is highly probable that it is integrated activity of these pumps which cause the demonstrated increase in intracellular pH. This rise is closely linked with increased motility and respiration of *Fucus serratus* spermatozoa, and evidence indicates that the increase in motility is directly mediated through activation of the dynein ATPase of the flagella. Any factor that increases the activity of this enzyme will increase the rate of respiration. All the results strongly suggest that respiration and motility of these spermatozoa are closely linked, most likely because the ATP produced by respiration is used primarily by the dynein ATPase.

Second messengers, have also been strongly implicated in the initiation/regulation of the motility and respiration of *Fucus serratus* spermatozoa. Indirect evidence with phosphodiesterase inhibitors and analogues of cAMP have shown that cAMP is involved and causes increases in motility. In most cells a rise in cAMP is triggered through the activation of adenylate cyclase and studies with forskolin indicate that this also may be the case in *Fucus serratus* spermatozoa. However, the first messenger which activates adenylate cyclase remains unknown, but a chemo-attractant released by *Fucus* eggs, similar to n-hexane, maybe the first

messenger however, investigations to ascertain this first messengers exact role in initiating/regulating sperm motility need to be undertaken. Further work needs to be done on spermatozoa from *Fucus serratus* to identify the phosphorylated protein products of a cAMP activated protein kinase.

Another second messenger system that appears to have a role in the regulation of sperm motility is the inositol phosphate pathway. Indirect evidence using activators and inhibitors have shown that protein kinase C may be involved in increasing the motility and respiration of *Fucus serratus* spermatozoa. One action of PKC which may be involved in spermatozoa of *Fucus serratus* is the phosphorylation and consequent activation of the Na^+/H^+ exchanger. This in turn would bring about intracellular alkalisation, thus activating the motility of spermatozoa. The activation of PKC activity could also regulate sperm motility through controlling the phosphorylation state of axonemal proteins. The existence of such a mechanism needs further investigation.

External Ca^{2+} is also important in activating the motility and respiration of *Fucus serratus* spermatozoa, and Ca^{2+} appears to enter spermatozoa through a voltage-dependent channel. A rise in external Ca^{2+} levels causes a corresponding increase in intracellular Ca^{2+} levels which correlates with an increase in the motility of *Fucus serratus* spermatozoa. Results in this study have also shown a link between a rise in pHi and an increase in the intracellular concentration of free Ca^{2+} but the exact mechanisms by which $[Ca^{2+}]i$ regulates the motility of spermatozoa remains unclear. There are many Ca^{2+} binding proteins in cells involved in a wide range of regulatory activities and further work is necessary to identify such proteins and their role in regulating motility in spermatozoa from *Fucus serratus*.

From the work presented it is clear that the mechanisms that regulate motility in spermatozoa from *Fucus serratus* are complex and involve a number of different factors. Many of these factors such as pH appear to be general to all spermatozoa so far studied. The importance of other factors, such as Na^+ and temperature, which are

critical for the activation of sperm from *Fucus serratus* may be related to the specialised surroundings in which fertilisation occurs. It is clear that the action of each of these factors needs to be studied intracellularly in order to fully evaluate the mechanisms by which they operate.

Appendices

Appendix 1.

Intracellular Free Ca^{2+} Concentration.

The concentration of intracellular free Ca^{2+} ($[Ca^{2+}]i$), using the fluorescent compound Fura-2-AM, was calculated using the equation of Grynkiewicz and colleagues (1985):

$$[Ca^{2+}]i = Kd \times (R - R_{min}) / (R_{max} - R)$$

Kd = the dissociation constant for the Fura-2-Ca^{2+} complex = 220 nM
R = the ratio of fluorescence emission obtained after excitation at 350 and 385 nm, of spermatozoa loaded with Fura-2-AM.
R_{min} = minimum fluorescence ratio of spermatozoa loaded with Fura-2-AM obtained in the presence of EGTA and manganese.
R_{max} = maximum fluorescence ratio of spermatozoa loaded with Fura-2-AM obtained in the presence of bromo A23187 and Ca^{2+}.

For example, Figure 2.IV which shows typical scans over the excitation range of 200 to 500 nm for spermatozoa of *Fucus serratus* loaded with Fura-2-AM in the presence or absence of Ca2+. The following values for fluorescence emission at 500 nm were obtained following excitation at 350 and 385 nm.

	Fluorescence Values	
	350	385
Spermatozoa loaded with Fura-2-AM (-Ca^{2+})	240	54.50
Spermatozoa loaded with Fura-2-AM + EGTA & manganese	165	68.75
Spermatozoa loaded with Fura-2-AM + Bromo A23187 & Ca^{2+}	310	36.90

$$R = \frac{240}{54.50} = 4.40$$

$$R_{min} = \frac{165}{68.75} = 2.40$$

$$R_{max} = \frac{310}{36.90} = 8.40$$

Therefore:

$$[Ca^{2+}]i = 220 \times (4.40 - 2.40) / (8.40 - 4.40)$$
$$= 220 \times \frac{2}{4}$$
$$= 220 \times 0.5$$
$$= 110 \text{ nM}$$

Thus the intracellular free Ca^{2+} concentration of spermatozoa of *Fucus serratus* suspended in ASW is 110 nM.

Appendix 2.

Abbreviations

Abbreviation	Name of Compound
AMP	Adenosine-5'-monophosphate
ASW	Artifical seawater
ATP	Adenosine-5'-triphosphate
BCECF	2',7'-bis-(-2-carboxyethyl)-5(and-6) carboxyfluorescein
BCECF-AM	2',7'-bis-(-2-carboxyethyl)-5(and-6) carboxyfluoresceinacetoxyl methylester
$[Ca^{2+}]i$	Intracellular concentration of free calcium
cAMP	Adenosine 3',5'-cyclic monophosphate
dbcAMP	Dibutryl adenosine 3',5' cyclic monophosphate
DMSO	Dimethyl sulfoxide
DNP	2,4-dinitrophenol
EDTA	Ethylene diaminetetraacetic acid
EGTA	Ethylene glycol-bis (β-amino-ethylether) N,N',N',N'-tetraacetic acid
FCCP	Carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone
Fura-2-AM	Fura-2- acetoxy methylester
H-7	I-(5-isoquinolinylsulfonyl)-2-methyl piperazine
Hepes	N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid

NAD^+	β-Nicotinamide-adenine dinuleotide, oxidised
NADH	β-Nicotinamide-adenine dinuleotide, reduced
OCaASW	Ca^{2+}-free artifical seawater
ONaASW	Na^+-free artifical seawater
Pi	Inorganic phosphate
β-PDD	4 β-phorbol 12, 13, didecanoate
pHi	Intracellular pH
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13 acetate
SITS	Acetamido 4' isothiocyanate stilbene 2,2'-disulfonic acid

Appendix 3.

Current Research

In the present study caudal epididymal spermatozoa showed some degree of motility. A recent report suggests that caudal epididymal spermatozoa in the rat are immotile and they become motile upon ejaculation (Armstrong *et al.*, 1994). It is probable that dilution of spermatozoa into Earles' medium, as carried out in our study stimulated motility, whereas undiluted spermatozoa in caudal epididymal fluid, as observed by the above authors, are immotile. These authors examined the role of intracellular signal transduction mechanisms in regulating the motility of epididymal spermatozoa. The addition of Ca^{2+} to immotile spermatozoa induced motility as did the addition of dibutryl cAMP, bicarbonate or the phosphodiesterase inhibitors caffeine and theophylline. These latter results suggested that the adenylate cyclase pathway is involved in the regulation of motility in rat caudal epididymal spermatozoa. These results are in agreement with our findings which suggest a role for Ca^{2+} and the adenylate cyclase pathway in the regulation of sperm motility both in rat epididymal spermatozoa and *Fucus serratus* spermatozoa. However, in contrast to our findings in rat epididymal spermatozoa Armstrong and colleagues (1994) showed that the addition of activators of protein kinase C (PKC), such as the phorbol diester PMA, failed to induce motility in rat caudal epididymal spermatozoa. These differences cannot be entirely explained by a dilution factor, as in our studies both diluted and undiluted spermatozoa responded to PMA with increased motility. Further studies using a variety of conditions are necessary to resolve these anomalies. In a recent study by Ashizawa and colleagues (1994a) on fowl spermatozoa the activators of PKC SC-9 and OAG inhibited sperm motility, and the inhibitor H-7 had no effect. Once again this is in contrast to our findings in the rat. In *Fucus serratus* the addition of phorbol diesters, known to activate PKC, also

stimulated the motility of spermatozoa and the addition of H-7 caused a decrease in the motility. Ashizawa and colleagues (1994a) also reported that the addition of SC-9 and OAG was also associated with a rise in the intracellular free Ca^{2+} concentration. It was not clear why the presence of these agents inhibited motility even though the intracellular free Ca^{2+} concentration increased, when in their absence such an increase stimulated motility (Ashizawa *et al.*, 1994a & b). In our study the activators of PKC also caused a rise in the intracellular free Ca^{2+} concentration in *Fucus serratus* spermatozoa and an inhibitor of PKC, H-7 caused a decrease in the level of intracellular free Ca^{2+}. Other workers (Rotem *et al.*, 1990a & b) have also shown that the addition of phorbol diesters, as well as OAG stimulate the motility of human spermatozoa. Thus it appears that activation of PKC may well have species specific effects on the regulation of sperm motility.

Calcium as a signal transducer was thought to have two main effects on mammalian spermatozoa. Firstly, it modulates flagella wave form and secondly, it has been shown to inhibit motility when present at micro-molar concentrations (Lindermann and Kanous, 1989). However, more recent studies have shown that it can activate motility at micro-molar concentrations (Ashizawa *et al.*, 1994b). Fowl spermatozoa, immobilised by raising the temperature from 30°C to 40°C, were restored to maximal motility by the addition of Ca^{2+}. Further, in the presence of the Ca^{2+} chelator, BAPTA/AM the motility of intact fowl spermatozoa was negligible at 30°C, a temperature at which these spermatozoa are normally active. The same authors showed that the addition of excess Ca^{2+} caused a rise in intracellular free Ca^{2+} coupled with stimulation of motility. Once again these results are in agreement with our finding for *Fucus serratus* spermatozoa and imply that a rise in the intracellular free Ca^{2+} concentration is vital for sperm motility. Calmodulin, the Ca^{2+}-binding protein involved in mediating the effect of Ca^{2+} on cellular processes (Means *et al.*, 1982) has also been shown to be present in spermatozoa (Garbers *et al.*, 1980). Ashizawa and colleagues (1994b) have shown that calmodulin is involved in the regulation of motility in fowl spermatozoa and this regulation is

downstream of Ca^{2+} entry. The addition of the calmodulin antagonists W-7 and trifluoperazine inhibited the motility of fowl spermatozoa even in the presence of Ca^{2+} and, although Ca^{2+} restored motility in BAPTA/AM treated spermatozoa, it did not do so in the presence of W-7 (Ashizawa *et al.*, 1994b). These authors also reported that both the oxygen uptake and ATP concentration of fowl spermatozoa are markedly lower in the presence of calmodulin antagonists, suggesting that the inhibition of motility of fowl spermatozoa observed in the presence of W-7 and trifluoperazine may be due to calmodulin-regulated energy depletion. Ashizawa and co-workers (1994b) have also suggested that calmodulin regulates sperm motility through stimulating adenylate cyclase and consequently increasing levels of cAMP, which is a prerequisite to the stimulation of sperm motility in most mammalian species.

As previously mentioned activation of sperm motility appears to be species specific. Other differences in the regulation of motility from, for example, fowl and mammals have been reported. Milli-molar concentrations of Ca^{2+} are required for maximal motility in fowl spermatozoa, whereas at this concentration Ca^{2+} inhibits the motility of mammalian spermatozoa (Ashizawa *et al.*, 1994b; Tash and Means, 1982). Cyclic AMP is required for the initiation and maintenance of motility in mammalian spermatozoa (Tash and Means, 1982; Linderman and Kanous, 1989), but it is not required to initiate fowl sperm motility (Ashizawa *et al.*, 1994a & b). In our study, on the motility of *Fucus serratus* spermatozoa, we have shown that as with fowl spermatozoa milli-molar concentrations of Ca^{2+} stimulate sperm motility. However, for these spermatozoa we have also shown, indirectly, that cAMP is necessary for the initiation and stimulation of motility. Thus the regulation of motility of spermatozoa appears to be unique to each species studied and the spermatozoa of *Fucus serratus* has similarities with mammalian spermatozoa, in that cAMP stimulates motility and PKC is also involved but, milli-molar concentrations of Ca^{2+} stimulate the motility of *Fucus serratus* spermatozoa in a similar manner to that observed with fowl spermatozoa.

References

Ashizawa, K., Katayama, S., Kabayashi, T., and Tsuzuki, Y. (1994a). Possible role of protein kinase C in regulation of flagellar motility and intracellular free Ca^{2+} concentration of fowl spermatozoa. J. Reprod. Fertil. **101** 511-517.

Ashizawa, K., Tomonaga, H., and Tsuzuki, Y. (1994b). Regulation of flagellar motility of fowl spermatozoa: evidence for the involvement of intracellular free Ca^{2+} and calmodulin. J. Reprod. Fertil. **101** 265-272.

Armstrong, V.L., Clulow, J., Murdoch, R.N., and Jones, R.C. (1994). Intracellular signal transduction mechanisms of rat epididymal spermatozoa and their relationship to motility and metabolism. Mol. Reprod. Dev. 38 77-84

Garbers, D.L., Hansborough, J.R., Radany, E.W., Hyne, R.V., and Kopf, G.S. (1980). Purification and characterization of calmodulin from sea urchin spermatozoa. J. Reprod. Fertil. **59** 377-381.

Lindemann, C.B., and Kanous, K.S. (1989). Regulation of mammalian sperm motility. Archives of Andrology **23** 1-22.

Means, A.R., Tash, j.S., and Chafouleas, J.G. (1982). Physiological implications of the presence, distribution and regulation of calmodulin in eukaryotic cells. Physiological Reviews **62** 1-39.

Rotem, R., Paz, G.F., Homonnai, Z.T., Kalina, M., and Naor, Z. (1990a). Protein kinase C is present in human sperm: Possible role in flagellar motility. Proc. Natl. Acad. Sci. USA **87** 7305-7308.

Rotem, R., Paz, G.F., Homonnai, Z.T., Kalina, M., and Naor, Z. (1990b). Further studies on the involvement of protein kinase C in human sperm flagellar motility. Endrocrinology **1** 2571-2577.

Tash, J.S., and Means, A.R. (1982a). Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol. Reprod. **26** 745-763.

REFERENCES

REFERENCES

Acott, T.S. and Carr, D.W. (1984). Inhibition of bovine spermatozoa by caudal epididymal fluid II. Interaction of pH and a quiescence factor. Biol. Reprod. **30** 926-935.

Adam, D.E., and Wei, J. (1975). Mass transport of ATP within the motile sperm. J. Theor. Biol. **49** 125-145.

Aitken, R.J., Irvine, S., and Kelly, R.W. (1986). Significance of intracellular calcium and cyclic adenosine 3',5' monophosphate in the mechanism by which postaglandins influence human sperm function. J. Reprod. Fert. **77** 451-462.

Amann, R.P., Hay, S.R., and Hammerstedt, R.H. (1982). Yield characteristics, motility and cAMP content of sperm isolated from several regions of ram epididmis. Biol. Reprod. **27** 723-733.

Anderson, J.M. (1989). Membrane-derived fatty acids as precursors to second messengers: In Plant growth and Development. Plant Biology Vol. 6 (Eds; Boss, W.F., and Morre, D.J.) Alan R Liss In. N.Y. USA 181-212.

Aronson, P.S. (1985). Kinetic properties of the plasma membrane Na^+/H^+ exchanger. Annu. Rev. Physiol. **47** 545-560.

Aronson, P.S., Nee, J., and Suhm, M.A. (1982). Modifier role of internal H^+ in activating the Na^+/H^+ exchanger in renal microvillas membrane vesicles. Nature **299** 161-163.

Ashizawa, K., Hashiquchi, A., and Tsuzuki, Y. (1992). Intracellular free Ca^{2+} concentration in fowl spermatozoa and its relationship to motility and respiration in spermatozoa. J. Reprod. Fert. **96** 395-405.

Atreja, S.K., and Anand, S.R. (1985). Phospholipase and lysophospholipase activation of goat spermatozoa in transit from caput to caudal epididmides. J. Reprod. Fertil. **74** 687-691.

Austin, C.R. (1951). Observations on the penetration of the sperm into the mammalian egg. Aust. J. Sci. Res. (B). **4** 581-596.

Austin, C.R., and Bishop, M.W.H. (1958). Role of the rodent acrosome and perforatorium in fertilization. In: Fertilization Proc. R. Soc. Lond. (Biol). **149** 241-248.

Babcock, D.F., (1988). Cross talk between voltage-dependent components of the signal transduction machinery of bovine spermatozoa. Biol. Reprod. **38** (Suppl. I) 60a.

Babcock, D.F., First, N.L., and Lardy, H.A. (1976). Action of Ionophore A23187 at the cellular level: Seperation of effect at the plasma and mitochondrial membranes. J. Biol. Chem. **251** 3881-3886.

Babcock, D.F., Rufo, C.A., and Lardy, H.A. (1983). Potassium-dependent increase in cytosolic pH stimulated metabolism and motility of mammalian sperm. Proc. Natl. Acad. Sci. U.S.A. **80** 1327-1331.

Babcock, D.F., and Pfeiffer, D.R. (1987). Independent elevation of cytosolic [Ca^{2+}] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. J. Biol. Chem. **262** 15041-15047.

Bansal, P., and Atreja, S.K. (1991). Phosphoinositide specific phospholipase C activity of goat spermatozoa in transit from the caput to the cauda epididymis. Indian Journal of Biochemistry and Biophysics **28** 307-311.

Bedford, J.M. (1970). Specific capacitation and fertilization in mammals. Biology of Reproduction (Suppl.). **2** 128-158.

Bedford, J.M. (1974). Report of a worjshop: Maturation of the fertilizing ability of mammalian spermatozoa in the male and female reproductive tract. Biol. Reprod. **11** 346-362.

Bedford, J.M. (1975). Maturation, transport and fate of spermatozoa in the epididymis. In: Handbook of Physiology-Endrocrinology **Vol. 5** American Physiological Society, Washington. D.C., 303-313.

Bedford, J.M. (1983). Significance of the need for sperm capacitation before fertilization in eutherian mammals. Biology of Reproduction. **28** 108-120.

Bedford, J.M., Calvin, H.I., and Cooper, G.W. (1973). The maturation of spermatozoa in the human epididymis. J. Reprod. Fertil. (Suppl.). **18** 199-213.

Benson, B.B., and Krause, Jr, D. (1984). The concentration and isotopic fractionation of oxygen dissolved in freshwater and sea water in equilibrium with the atmosphere. Limnol. Oceanogr. **29** 620-632.

Berkaloff, C., and Rousseau, B. (1979). Ultrastructure of male gametogensis in *Fucus serratus* (Phaeophyceae). J. Phycology **15** 163-173.

Berridge, M.J. (1984). Inositol triphosphate and diacylglycerol as second messengers. Biochem. J. **220** 345-360.

Berridge, M.J. (1987a). Inositol triphosphate and diacylglycerol: Two interacting second messengers. Ann. Rev. Biochem. **56** 159-193.

Berridge, M.J. (1987b). Inositol triphosphate as a second messenger in signal transduction. Ann. N.Y. Acad. Sci. **494** 39-51.

Bibring, T., Bascandall, J., and Harter, C.C. (1984). Sodium-dependent pH regulation in active sea urchin sperm. Dev. Biol. **101** 425-435.

Blowers, D.P., and Trewavas, A.J. (1989). Rapid cycling of autophosphorylation of a Ca^{2+}-calmodulin regulated plasma membrane located protein kinase from pea. Plant Physiology **90** 1279-1285.

Boron, W.F., McCormick, W.C., and Roos, A. (1979). pH regulation in barnacle muscle fibers: dependence on intracellular and extracellular pH. Am. J. Physiol. **237** 185-193.

Bradley, M.P., and Forrester, I.T. (1980). A sodium-calcium exchange mechanism in plasma membrane vesicles isolated from ram sperm flagella. FEBS Lett., **121** 15-18.

Brandt, H., Acott, T.S., Johnson, D.J., and Hoskins, D.D. (1978). Evidence for an epididymal origun of bovine sperm forward motility protein. Biol. Reprod., **19** 830-835.

Brawley, S.H., and Bell, E. (1987). Partial activation of *Fucus* eggs with calcium ionophores and low sodium seawater. Developmental Biology **122** 217-226.

Breitbart, H., Rubinstein, S., and Nass-Arden, L. (1985). The role of calcium and Ca^{2+}-ATPase in maintaining motility in ram spermatozoa. J. Biol. Chem. **260** 11548-11554.

Breitbart, H., Cragoe. Jnr, E.J., and Lardy, H.A. (1990). Stimulation of Ca^{2+} uptake into epididymal bull spermatozoa by analogues of amiloride. Eur. J. Biochem. **192** 529-535.

Brokaw, C.J. (1961). Movement and nucleoside polyphosphatase activity of isolated flagella from Polytoma avella. Exp. Cell Res. **22** 151-162.

Brokaw, C.J. (1987). regulation of sperm flagellar motility by calcium and cAMP-dependent phosphorylation. J. Cell Biochem. **35** 175-184.

Brokaw, C.J., and Benedict, B. (1968). Mechanochemical coupling in flagella I. Movement-dependent dephosphorylation of ATP by glycerated spermatozoa. Arch. Biochem. **125** 770-778.

Brostrom, C.O., Huang, Y.C., Breckenridge, B.M., and Wolff, D. (1975). Identification of a calcium-binding protein as a calcium-dependent regulator of brain adenylate cyclase. Proc. Natl. Acad. Sci. USA **72** 62-68.

Brownlee, C. (1989). Visualizing cytoplasmic calcium in polarizing zygotes and growing rhizoids of *Fucus serratus*. Biol. Bull. **176(S)** 14-17.

Callow, J.A., Callow, M.E., and Evans, L.V. (1985). Fertilization in *Fucus*. In: Biology of Fertilization Vol. 2 Academic Press 389-407.

Carr, D.W., and Acott, T.S. (1989). Intracellular pH regulates bovine sperm motility and protein phosphorylation. Biology of Reproduction. **41** 907-920.

Casillas, E.R., and Hoskins, D.D. (1970). Activation of monkey spermatozoal adenyl cyclase by thyroxine and triiodothyronine, Biochem. Biophys. Res. Commun. **40** 255-262.

Castagna, M., Takai, Y., Kabuahi, K., Sano,K., Kikkawa, U., and Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. **257** 7847-7851.

Chance, B., and Williams, G.R. (1956). Respiration. Adv. Enzymol. **17** 65-134.

Chang, M.C. (1951). Fertilizing capacity of spermatozoa deposited in Fallopian tibes. Nature. **168** 997-998.

Christen, R., Schackmann, R.W., and Shapiro, B.M. (1982). Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin, *Strongylocentrotus purpuratus*. J. Biol. Chem. **257** 14881-14890.

Christen, R., Schackmann, R.W., and Shapiro, B.M. (1983). Metabolism of sea urchin sperm. J. Biol. Chem. **258** 5392-5399.

Clapper, D.L., and Epel, D. (1981). Isolation and utilization of a sperm motility initiating peptide in the horseshoe crab, *Limulus polyphemus*: Evidence for involvement of Ca^{2+} but not intracellular pH or membrane potential in motility initiation. J. Cell Biol. **91** 179a (abstract).

Cooke, A.E., Elvidge, J.A., and Bently, R. (1951). Fertilisation in the Fucaceae: investigations on the nature of the chemotactic substance produced by eggs of *Fucus serratus* and *F. vesiculosus*. Proc. R. Soc. london, Ser. B. **138** 97-114.

Dan, J.C. (1954). Studies on the acrosome III. Effect of calcium deficiency. Biol. Bull. **107** 335-349.

Darszon, A., García-Soto, J., González-Mortinez, M., Guerrero, A., Sánchez, J.A., and Lievano, A. (1987). Ion transport mechanisms in sea urchin sperm. In: New horizons in sperm cell research (Ed; Mohri, H.) Japan Scientific Society Press, Yokyo/Gordon and Breech Scientific Publishers. New York 169-183.

Del Rio, A.G., and Raisman, R. (1978). cAMP in spermatozoa taken from different segments of rat epididymis. Experientia **34** 670-671.

De Jonge, C.J., Han, H.L., Mach, S.R., and Zaneveld, L.J. (1991). Effect of phorbol diesters, synthetic diacylglycerols and a protein kinase C inhibitor on the human sperm acrosome reaction. J. Androl. **12** 62-70.

Drobak, B.K. (1993). Phosphoinostides and protein phosphorylation in plant signal transduction. Cell Biology **4** 123-130.

Dunham, P.B., and Sengh, O. (1977). Lithium efflux through the Na/K pump in human erthrocytes. Proc. Natl. Acad. Sci. USA. **74** 3099-3103.

Evans, L.V., Callow, J.A., and Callow, M.E. (1982). The biology and biochemistry of reproduction and early development in *Fucus*. In: Progress in Phycological Research (Round, F.E., and Chapman, D.J. eds) Elvservier, Amsterdam. Vol. 1 67-110.

Feng, B., Bhattacharyya, A., and Yanagimachi, R. (1988). Ca^{2+} is essential for the motility of plasma membrane-intact, but not of demembranated hamster spermatazoa. Andrologia **20** 155-162.

Fraser, L.R. (1993). Calcium channels play a pivotal role in the sequence of ionic changes involved in initiation of mouse sperm acrosomal exocytosis. Molecular Reproduction and Development **36** 368-376.

Fraser, L.R. (1994). Personnel correspondence.

Friedmann, I. (1961). Cinemicography of spermatozoids and fertilization in Fucales. **Bull. Res. Counc. Isr. 10D** 73-83.

Garbers, D.L., Lust, W.D., First, N.L., and Lardy, H.A. (1971). Effects of phosphodiesterase inhibitors and cyclic nucleotides on sperm respiration and motility. Biochemistry. **10** 1825-1831.

Garbers, D.L., and Kopf, G.S. (1980). The regulation of spermatozoa by calcium and cyclic nucleotides. Adv. Cyclic Nucleotide Res. **13** 251-306.

Garbers, D.L., Hansborough, J.R., Radany, E.W., Hyne, R.V., and Kopf, G.S. (1980). Purification and characterization of calmodulin from sea urchin spermatozoa. J. Reprod. Fertil. **59** 377-381.

Garbers, D.L., Tubb, D.J., and Hyne, R.V. (1982). A requirement of bicarbonate for Ca^{2+}-induced elevation of cyclic AMP in guinea pig spermatozoa. J. Biol. Chem. **257** 8980-8984.

Garbers, D.L., Kopf, G.S., Tubb, D.J., and Olson, G. (1983). Elevation of sperm adenosine 3',5' monophosphate concentration by fucose-sulfate-rich complex associated with eggs. I. Structural characterization. Biol. Reprod. **29** 1211-1220.

García-Soto, J., de De la Torre, L., and Darszon, A. (1985). Increasing intracellular pH of sea urchin sperm with NH_4Cl induces Ca^{2+} uptake and acrosome reaction in the absence of egg jelly. FEBS Lett. **190** 33-36.

García-Soto, J., Gonzálaz-Mortinez, M., de De la Torre, L., and Darszon, A. (1987). Internal pH can regulate Ca^{2+} uptake and the acrosome reaction in sea urchin sperm. Dev. Biol. **120** 112-120.

Garty, N.B., Galini, D., Aharonheim, A., Ho, Y.K., Phillips, D.M., Dekel, N., and Salomon, Y. (1988). G-protein in mammalian gametes: an immocytochemical study. J. Cell Sci. **91** 21-31.

Gatti, J.L., and Christen, R. (1985). Regulation of internal pH of sea urchin sperm: A role for the Na/K pump. J.Biol. Chem. **260** 7599-7602.

Gibbons, B.H. (1980). Intermittent swimming of live sea urchin sperm. J. Cell Biol. **84** 13-27.

Gibbons, I.R., (1982). Introduction: Dynein ATPases. Prog. Clin. Biol. Res. **80** 87-93.

Gibbons, B.H., and Gibbons, I.R. (1972). Flagellar movement and adenosine triphosphate activity in sea urchin sperm extracted with triton X-100. J. Cell Biol. **54** 75-97.

Gibbons, B.H., and Gibbons, I.R. (1973). Calcium-induced quiescence in reactivated sea urchin sperm. J. Cell Biol. **13** 337-357.

Gibbons, B.H., Ogawa, K., and Gibbons, I.R. (1976). The effect of antidynein 1 serum on the movement of reactivated sea urchin sperm. J. Cell Biol. **71** 823-831.

Gibbons, I.R., Cosson, M.P., Evans, J.A., Gibbons, B.H., Houch, B., Martinson, K.H., Sale, W.S., and Tang, W.S.Y. (1978). Potent inhibition of dynein adenosine triphosphatase and of the motility of cilia and sperm flagella by vanadate. Proc. Natl. Acad. Sci. USA. **75** 2220-2224.

Goldstein, S.F. (1979). Starting transients in sea urchin sperm flagella. J. Cell Biol. **80** 61-68.

Goltz, J.S., Gardener, T.K., Kanous, K.S., and Lindermann, C.B. (1988). The interaction of pH and cyclic adenosine 3',5' monophosphate on activation of motility in triton X-100 extracted bull sperm. Biology of Reproduction. **39** 1129-1136.

Gray, J.P., Hardman, J.G., Hammer, J.L., Hoos, R.T., and Sutherland, E.W. (1971). Adenyl cyclase, phosphodiesterase and cyclic AMP of human sperm and seminal plasma (Abstract) Feberation Proc. **30** 1267.

Grynkiewicz, G., Poenie, M., and Tsien, R. (1985). A new generation of Ca^{2+} indicators with greatly improved fluoresence properties. J. Biol. Chem. **260** 3440-3450.

Hansbrough, J.R., and Garbers, D.L. (1981). Sodium-dependent activation of sea urchin spermatozoa by speract and monensin. J. Biol. Chem. **256** 1447-1452.

Harumi, T., Hoshino, K., and Suzukei, N. (1992). Effects of sperm-activating peptide I on *Hemicentiotus pulcherrimus* spermatozoa in high potassium sea water. Develop. Growth and Differ. **34** 163-172.

Hepler, P.K., and Wayne, R,O. (1985). Calcium and plant development. Ann. R. Plants **36** 397-439.

Hetherington, A., and Trewavas, A. (1982). Calcium-dependent protein kinase in pea shoot membranes. FEBS Lett. **145** 67-71.

Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry **23** 5036-5041.

Hino, A., Hiruma, T., Fujiwara, A., and Yasumasu, I. (1980). Inhibition of respiration in sea urchin spermatozoa following interaction with fixed unfertilized eggs.
IV. State 4 respiration in spermatozoa of *Hemicentrotus Pulcherrimus* after their interaction with fixed unfertilized eggs. Development Growth and Differenition. **22** 813-820.

Hlubeck, J.R., Hora, J., Tombe, T.P., and Weeden, B.C.L. (1970). The gamone of *Fucus serratus*. Tetrahedron Letters. **59** 5163-5164.

Hoffmann, E.K. (1986). Anion transport systems in the plasma membrane of vertebrate cells. Biochim. Biophys. Acta **864** 1-31.

Horowitz, J.A., Toeg, H., and Orr, G.A. (1984). Characterization and localization of cAMP-dependent protein kinase in rat caudal epididymal sperm. J. Biol. Chem. **259** 832-838.

Horowitz, J.A., Voulales, P., Wasco, W., MacLeod, J., Paupard, M.C., and Orr, G.A. (1989). Biochemical and immunological characterization of the flagellar-associated regulatory subunit of type II cyclic adenosine 3',5'-monophosphate-dependent protein kinase. Arch. Biochem. Biophys. **270** 411-418.

Hoskins, D.D. (1973). Adenosine nucleotide mediation of fructolysis and motility by bovine epididymal spermatozoa. J. Biol. Chem. **248** 1135-1140.

Hoskins, D.D., Casillas, E.R., and Stephens, D.T. (1972). Cyclic AMP-dependent protein kinase of bovine epididymal spermatozoa. Biochem. Biophys. Res. Commun. **48** 1131-1138.

Hoskins, D.D., Stephens, D.T., and Hall, M.L. (1974). Cyclic adenosine 3',5' monophosphate and protein kinase levels in developing bovine spermatozoa. J. Reprod. Fertil. **37** 131-133.

Hoskins, D.D., and Casillas, E.R. (1975). Function of cyclic nucleotides in mammalian spermatozoa. In Handbook of Physiology- Endrocrinology V Chapter 22 453-460.

Hoskins, D.D., Hall, M.L., and Munsterman, D. (1975). Induction of motility in immature bovine spermatozoa by cyclic AMP phosphodiesterase inhibitors and seminal plasma. Biology of Reproduction. **13** 168-176.

Hoskins, D.D., Brandt, H., and Acott, T.S. (1978). Initiation of sperm motility in the mammalian epididymis. Fed. Pro. **37** 2534-2542.

Hyne, R.V., and Garbers, D.L. (1979a). Regulation of guinea pig sperm adenylate cyclase by calcium. Biol. Reprod. **21** 1135-1142.

Hyne, R.V., and Garbers, D.L. (1979b). Calcium-dependent increase in adenosine 3',5' monophosphate and induction of the acrosome reaction in guinea pig spermatozoa. Proc. Natl. Acad. Sci. U.S.A. **76** 5699-5703.

Ishiquro, K., Murofushi, H., and Sahai, H. (1982). Evidence that cAMP-dependent protein kinase and a protein factor are involved in reactivation of Triton X-100 models of sea urchin and starfish spermatozoa. J. Cell Biol. **92** 777-782.

Ives, H.E., Yee, V.J., and Warnock, D.G. (1983). Mixed type inhibition of the renal Na^+/H^+ antiporter by Li^+ and amiloride. J. Biol. Chem. **258** 3503-3508.

Johnson, J.D., and Epel, D. (1976). Intracellular pH and activation of sea urchin eggs after fertilization. Nature **262** 661-664.

Kaczorowski, G.J., Barros, F., Dethmers, J.K., Trumble, M.J., and Cragoe Jnr, E.J. (1985). Inhibition of Na^+/Ca^{2+} exchange in pituitory plasma membrane vesicles by analogues of amiloride. Biochemistry **24** 1394-1403.

Katz, D.F., and Overstreet, J.W. (1981). Sperm motility assessment by videomicrography. Fertil. Steril. **35** 188-193.

Katz, D.F., Drobnis, E.Z., and Overstreet, J.W. (1989). Factors regulating mammalian sperm migration through the female reproduction tract and oocyte vestments. Gamete Res. **22** 443-469.

Kimura, K., Katoh, N., Sakurada, K., and Kubo, S. (1984). Phospholipid sensitive Ca^{2+}-dependent protein kinase system in testis: localization and endogenous substrates. Endocrinology **115** 2391-2399.

L'Allemain, G., Paris, S., and Poussegur, J. (1985). Role of a Na^+-dependent Cl^-/HCO_3^- exchange in regulation of intracellular pH in fibroblasts. J. of Biol. Chem. **260** 4877-4883.

Langer, G.A. (1982). Sodium-calcium exchange in the heart. Annu. Rev. Physiol. **44** 435-449.

Lardy. H.A., and Phillips, P.H. (1941). Phospholipids as a source of energy for motility of bull spermatozoa. Am. J. Physiol. **134** 542-548.

Lee, H.C. (1984a). Sodium and proton transport in flagella isolated from sea urchin spermatozoa. J. Biol. Chem. **259** 4957-4963.

Lee, H.C. (1984b). A membrane potential-sensitive Na^+-H^+ exchange system in flagella isolated from sea urchin spermatozoa. J Biol. Chem. **259** 15315-15319.

Lee, H.C. (1985). The voltage-sensitive Na^+/H^+ exchange in sea urchin spermatozoa flagellar membrane vesicles studied with an entrapped pH probe. J. Biol. Chem. **260** 10794-10799.

Lee, H.C., Breitbart, H., and Forte, J.C. (1980). The functional role of K^+-ATPase in proton transport by gastic microsomal vesicles. Ann. N.Y. Acad. Sci. **341** 297-311.

Lee, H.C., Forte, J.G., and Epel, D. (1982). In: Intracellular pH, its measurements, regulation and ultilization in cellular function. (Nuccitelli, R., and Deamers, D.W., eds). Allan Liss, New York. 136-180.

Lee, H.C., Johnson, C., and Epel, D. (1983). Changes in internal pH associated with initiation of motility and acrosome reaction of sea urchin sperm. Dev. Biol. **95** 31-45.

Lee, M.Y., and Iverson, R.M. (1976). An adenosine 3',5' monophosphate-dependent protein kinase from sea urchin spermatozoa. Biochim. Biophys. Acta. **429** 123-136.
Lindermann, C.B. (1978). A cAMP-induced increase in the motility of demembranated bull sperm models. Cell **13** 9-18.

Lindemann, C.B. (1978). A cAMP-induced increase in the motility of demembranated bull sperm models. Cell **13** 9-18.

Lindemann, C.B., and Rikmenspoel, R. (1972). Sperm flagella: Autonmous oscillations of the contractile system. Science **175** 337-338.

Lowry,O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **193** 265-275.

MacEwen, D.J., Mitchell, R., Johnson, M.S., Thomson, F.J., Lutz, E.M., Clegg, R.A., and Connor, K. (1993). Evidence that protein kinase Cα has reduced affinity towards 1,2-dioctanoyl-sn-glycerol: the effects of lipid activators on phorbol ester binding and kinase activity. European Journal of Pharmacology- Molecular Pharmacology Section **246** 9-18.

Majumder, G.C., Dey, C.S., Halder, S., and Barua, M. (1990). Biochemical parameters of initiation and regulation of sperm motility. Archives of Andrology. **24** 289-303.

Manton, I., and Clarke, B. (1956). Observations with the electron microscope on the internal structure of spermatozoid of *Fucus*. J. Exp. Bot. **7** 416-432.

Manton, I. (1969). Plant spermatozoids. In: Comparative spermatology, Proceedings of the International Symposium (Baccetti, B., ed). Academic Press, New York. 143-158.

Marme, D. (1989). The role of calcium and calmodulin in signal transduction. In: Second Messengers. In: Plant growth and Development. Plant Biology Vol. 6 (Eds; Boss, W.F., and Morre, D.J.) Alan R Liss In. N.Y. USA 57-80.

Mazorow, D.L., and Millar, D.B. (1990). Quinn 2 and Fura 2 measure calcium differently. Analytical Biochemistry **186** 28-30.

McCully, M.E. (1968). Histological studies on genus *Fucus* II. Histology of the reproductive tissues. Protoplasma. **66** 205-230.

Meech, R.W., and Thomas, R.C. (1980). Effect of measured calcium chloride injection on the membrane potential and internal pH of snail neurons. J. Physiol (London) **298** 111-129.

Mita, M. (1984). Activation of respiration in sea urchin spermatozoa by calcium ionophore A23187. Comp. Biochem. Physiol. (A). **77** 689-692.

Mohri, H. (1993). Role of tubulin and dynein in spermatozoan motility. Molecular reproduction and Development **36** 221-223.

Mohri, H., and Yanagimachi, R. (1980). Characteristics of motor apparatus in testicular, epididymal and ejaculated spermatozoa. A study using demembranated sperm models. Exp. Cell Res. **127** 191-196.

Morton, B., Harrigan-Lum, J., Albagli, L., and Jones, T. (1974). The activity of motility in quiescent hamster sperm from epididymis by calcium and cyclic nucleotides. Biochem. Biophys. Res. Commun. **56** 372-379.

Morisawa, S., Ishida, K., Okuno, m., and Morisawa, M. (1993). Roles of pH and cyclic adenosine monophosphate in the acquisition of potential for sperm motility during migration from the sea to the river in Chum Slmon. Molecular Reproduction and Development **34** 420-426.

Nuccitelli, R., and Deamer, D. (1982). Intracellular pH: its measurement, regulation and utilization in cellular functions. (Nuccitelli, R., and Deamer, D. eds) 100-131. Alan R. Liss N. York.

Müller, D.G. (1979). Olefinic hydrocarbons in seawater: Signal molecules for sexual reproduction in brown algae. Pure and Appl. Chem. **51** 1885-1891.

Müller, D.G., and Jaenicke, L. (1973). Fucoserraten, the female sex attractant of *Fucus serratus* L. (Phaephyta). FEBS Lett. **30** 137-139.

Müller, D.G., and Seferiadis, K. (1977). Specificity of sexual chemotaxis in *Fucus serratus* and *Fucus vesiclosus* (Phaeophyceae). Z. Pflazenphysiol **84** 85-94.

Nishioka, D., and Cross, N. (1978). Role of external sodium in sea urchin fertilisation. In Cell Reproduction (Dirsken, E.R., Prescott, D., and Fox, D.F. eds) 403-413. Academic Press N. York.

Nishizuka,Y. (1986). Studies and perspective of protein kinase C. Science **233** 305-312.

Ohtake, H. (1976a). Respiratory behaviour of sea urchin spermatozoa. I. Effect of pH and egg water on the respiratory rate. J. Experimental Zoology. **198** 303-312.

Ohtake, H. (1976b). Respiratory behavior of sea urchin spermatozoa. II. Sperm activating substance obtained from jelly coat of sea urchin eggs. J. Experimental Zoology. **198** 313-322.

Okamura, N., Tajima, Y., Soejima, A., Masuda, H., and Sugita, Y. (1985). Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase, J. Biol. Chem. **260** 9699-9705.

Okamura, N., Tanba, N., Fukuda, A., Sugita, Y., and Nagai, T. (1993). Forskolin stimulates porcine sperm capacitation by increasing calcium uptake. FEBS Lett. **316** 283-286.

Okuno, M. (1980). Inhibition and relaxation of sea urchin sperm flagella by vanadate. J. Cell Biol. **85** 712-725.

Okuno, M., and Brokaw, C.J. (1981). Effects of AMPPNP and vanadate on the mechanochemical cross bridge cycle in flagella. J. Muscle Res. Cell Motil. **2** 131-140.

Olson, G.E., and Danzo, B.J. (1981). Surface changes in rat spermatozoa during epididymal transit. Biology of Reproduction. **24** 431-443.

Paris, S., and Pouysseger, J. (1983). Biochemical characterization of the amiloride-sensitive Na^+/H^+ antiport in Chinese hamster lung fibroblasts. J. Biol. Chem. **258** 3503-3508.

Powell, H.T. (1963). Speciation in the genous *Fucus L.*, and releated genera. Syst. Assoc. Publ. **5** 63-77.

Pressman, B.C. (1976). Biological application of ionophores. Annu. rev. Biochem. **45** 501-530.

Quest, A.F., and Shapiro, B.M. (1991). Membrane association of flagellar creatine kinase in the sperm phosphocreatine shuttle. J. Biol. Chem. **266** 19803-19811.

Rasmussen, H. (1970). Cell communication, calcium ion, and cyclic adenosine monophosphate. Science **170** 402-412.

Reed, P.W., and Lardy, H.A. (1972). A23187: a divalent cation ionophore. J. Biol. Chem. **247** 6970-6977.

Repaske, D.R., and Garbers, D.L. (1983). A hydrogen ion flux mediates stimulation of respiratory activity by speract in sea urchin spermatozoa. J. Biol. Chem. **258** 6025-6029.

Rink, T. J. (1987). Intracellular pH and cytoplasmic free Ca^{2+}. Nature **327** 375-376.

Rink, T.J., Sanchez, A., and Hallam, T.J. (1983). Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. Nature **305** 317-319.

Rink, T.J., and Pozzan, T. (1985). Using quin-2 in cell suspension. Cell Calcium **6** 133-144.

Robison, G.A., Butcher, R.W., and Sutherland, E.W. (1968). Cyclic AMP. Annual Review of Biochemistry **37** 149-174.

Rojas, F.J., Bruzzone, M.E., and Moretti-Rojas, I. (1992). Regulation of cyclic adenosine monophosphate synthesis in human ejaculated spermatozoa II. The role of calcium and bicarbonate ions on the activation of adenylyl cyclase. Human Reproduction **7** 1131-1135.

Roos, A., and Boron, W.F. (1981). Intracellular pH. Physiol. Rev. **61** 296-434.

Rotem, R., Paz, G.F., Homonnai, Z.T., Kalina, M., and Naor, Z. (1990a). Protein kinase C is present in human sperm: Possible role in flagellar motility. Proc. Natl. Acad. Sci. USA **87** 7305-7308.

Rotem, R., Paz, G.F., Homonnai, Z.T., Kalina, M., and Naor, Z. (1990b). Further studies on the involvement of protein kinase C in human sperm flagellar motility. Endrocrinology **1** 2571-2577.

Rotem, R., Paz, G.F., Homonnai, Z.T.., Kalina, M., Lax, J., Breitbart, H., and Noar, Z. (1992). Ca^{2+}-independent induction of acrosome reaction by protein kinase C in human sperm. Endocrinology **131** 2235-2243.

Rottenberg, H. (1979). The measurement of membrane potential and delta pH in cells, organelles, and vesicles. Methods Enzymol. **55** 547-569.

Rufo, G.A., Schoff, P.K., and Lardy, H.A. (1984). Regulation of calcium content in bovine spermatozoa. J. Biol. Chem. **259** 2547-2552.

Schackmann, R.W., Eddy, E.M., and Shapiro, B.M. (1978). The acrosome reaction of *Strongyrocentrotus purpuratus* sperm. Ion requirements and movements. Dev. Biol. **65** 483-495.

Schackmann, R.W., Christen, R., and Shapiro, R.M. (1981). Membrane potential depolarization and increased intracellular pH accompany the acrosome reaction of sea urchin sperm. Proc. Natl. Acad. Sci. USA **78** 6066-6070.

Schafer, A., Bygrave, F., Matzenau, S., and Marme, D. (1985). Identification of a calcium-dependent and phospholipid-dependent protein kinase in plant tissue. FEBS Lett. **187** 25-28.

Schoenfield, C., Amelar, R.D., and Dubin, L. (1973). Stimulation of ejaculated human spermatozoa by caffeine. A preliminary report. Fertil. Steril. **24** 772-775.

Schoff, P.K., Cheetham, J., and Lardy, H.A. (1989). Adenylate Kinase activity in ejaculated bovine sperm flagella, J. Biol. Chem. **264** 6086-6091.

Seamon, K., and Daly, J.W. (1981a). Activation of adenylate cyclase by the diterpene forskolin does not require the quanine nucleotide regulatory protein. J. Biol. Chem. **256** 9799-9801.

Seamon, K., and Daly, J.W. (1981b). Forskolin: A unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res. **7** 201-224.

Shapiro, B.M., Schackman, R.W., and Garbel, C.A. (1981). Molecular approaches to the study of fertilization. Annu. Rev. Biochem. **50** 815-843.

Shapiro, B.M., Cook, S., Quest, A.F., Oberdorf, J., and Wothe, D. (1990). Molecular mechanisms of sea urchin sperm activation before fertilisation. J. Reprod. Fertil. Suppl. **42** 3-8.

Siffert, W., and Akkerman, J.W.N. (1988). Protein kinase C enhances Ca^{2+} mobilization in human platelets by activating Na^{+}/H^{+} exchange. J. Biol. Chem. **263** 4223-4227.

Simpson, A.M., Swan, M.A., and White, I.G. (1986). Action of phosphatidylcholine in protecting ram sperm from cold shock. Gamete Res. **15** 43-56.

Simpson, A.M., Swan, M.A., and White, I.G. (1987). Calcium uptake, respiration and ultrastructure of sperm exposed to ionophore A23187. Archives of Andrology **19** 5-18.

Stengel, D., and Hanoune, J. (1984). The sperm adenylate cyclase. Ann N.Y. Acad. Sci. **438** 283-286.

Sutherland, E.W., Robison, G.A., and Butcher, R.W. (1968). Some aspects of the biological role of adenosine 3',5' cyclic monophosphate (Cyclic AMP). Circulation **3** 279-306.

Takei, G.H., Flemming, A.D., and Yanagimachi, R. (1984). Phospholipids in guinea pig spermatozoa before and after capacitiation in vitro. Andrologia **16** 38-47.

Tash, J.S., and Means, A.R. (1982a). Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol. Reprod. **26** 745-763.

Tash, J.S., and Means, A.R. (1982b). Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate. Biol. Reprod. **28** 75-104.

Tash, J.S., and Means, A.R. (1983). Cyclic adenosine 3',5'-monophosphate, calcium and protein phosphorylation on flagella motility. Biol. Reprod. **28** 75-104.

Tash, J.S., and Means, A.R. (1987). Ca^{2+} regulation of sperm axonemal motility. Methods of Enzymology **139** 808-815.

Tash, J.S., and Means, A.R. (1988). cAMP-dependent regulatory processess in the acquisistion and control of sperm flagellar movement: Meiotic Inhibition: Molecular Control of Meiosis. 335-355.

Tash, J.S., Katarr, S.S., and Means, A.R. (1984). Flagellar motility requires the cAMP-dependent phosphorylation of a heat-stable NP-40 soluble 50-kD protein, axokinin. Cell. **38** 551-559.

Tash, J.S., Hidaka, H., and Means, A.R. (1986). Axokinin phosphorylation by cAMP-dependent protein kinase is sufficient for activation of sperm flagellar motility. J. Cell Biol. **103** 649-655.

Tash, J.S., Krinks, M., Patel, J., Means, R.L., Klee, C.B., and Means, A.R. (1988). Identification, characterization and functional correlation of calmodulin-dependent protein phosphatase in sperm. J. Cell Biol. **106** 1625-1633.

Thomas, P., and Meizel, S. (1988). An influx of extracellular calcium is required for initiation of the human sperm acrosome reaction induced by human follicular fluid. Gamete Res. **20** 397-411.

Tombes, R.M., and Shapiro, B.M. (1985). Metabolite channeling: a phosphorylcreatine shuttle to mediate high energy phosphate transport between sperm mitochondrion and tail. Cell **41** 325-334.

Tombes, R.M., and Shapiro, B.M. (1989). Energy transport and cell polarity: Relationship of phosphagen kinase activity to sperm function. J. Exp. Zool. **251** 82-90.

Tombes, R.M., Brokaw, C.J., and Shapiro, B.M. (1987). Creatine kinase dependent energy transport in sea urchin spermatozoa. Flagella wave altenuation and theoretical analysis of high energy phosphatase diffusion. Biophys. J. **52** 75-86.

Trimmer, J., Schackerman, R.W., and Vacquier, V.D. (1986). Monoclonal antibodies increases intracellular calcium in sea urchin spermatozoa. Proc. Natl. Acad. Sci. U.S.A.**83** 9055-9059.

Tse, C-M., Levine, S.A., Chis-Y, C.H., Brant, S.R., Pouyssegur, J., Montrose, M.H., and Donowitz, M. (1993). Functional characteristics of a cloned epithelical Na^+/H^+ exchanger (NHE3): resistance to amiloride and inhibition by protein kinase C. Proc. Natl. Acad. Sci. USA **90** 9110-9114.

Tsien, R.Y. (1980). New calcium indicator and buffer with high selectivity against magnesium and protons: Design synthesis, and properties of proto type structures. Biochemistry **19** 2390-2404.

Tsien, R.Y. (1981). A non-disruptive technique for loading calcium buffers and indicators into cells. Nature **290** 527-528.

Tsien, R.Y., Pozzon, T., and Rink, T.J. (1982). Calcium homeostasis in intact lymphocytes: Cytoplasmic free calcium monitored with new intracellularly trapped fluorescent indicators. J. Cell Biol. **94** 325-334.

Vijayaraghavan, S., Critochlow, L.M., and Hoskins, D.D. (1985). Evidence for a role for cellular alkalinization in the cyclic adenosine 3',5' monophosphate mediated initiation of motility in bovine caput spermatozoa. Biology of reproduction. **32** 489-500.

Vijayaraghavan, S., and Hoskins, D.D. (1986). Regulation of bovine sperm motility and cyclic adenosine 3',5,-monophosphate by adenosine and its analogues. Biol. Reprod. **34** 468-477.

Vijayaraghavan, S., and Hoskins, D.D. (1989). Quantitation of bovine sperm cytoplasmic calcium with Quin-2 and Fura-2: Evidence that external calcium does not have direct assess to the sperm cytoplasm. Cell Calcium. **10** 241-253.

Vijayaraghavan, S., and Hoskins, D.D. (1990). Changes in the mitochondrial calcium influx and efflux properties are responsible for the decline in sperm calcium during epididymal maturation. Molecular Reproduction and Development. **25** 186-194.

Visconti, P.E., and Tezon, J.G. (1989). Phorbol esters stimulates cyclic adenosine 3',5' monophosphate accumulation in hamster during in vitro capacitiation. Biol. Reprod. **40** 223-231.

Visconti, P.E., Muschietta, J.P., Flauna, M.M., and Tezon, J.G. (1990). Bicarbonate dependence of cAMP accumulation induced by phorbol esters in hamster spermatozoa. Biochimica et Biophysica Acta **1054** 231-236.

Voglmayr, J.K. (1975). Metobolic changes in spermatozoa during epididymal transit. In: Handbook of Physiology. Sect. 7, Vol. 5. (Hamilton, D.W., and Greep, R.O. eds.) Bethseda, MD: American Physiology Society 437-451.

Wasco, W.M., and Orr, G.A. (1984). Function of calmodulin in mammalian sperm: presence of a calmodulin-dependent cyclic nucleotide phosphodiesterase associated with demembranated rat caudal epididymal sperm. Biochem. Biophys. Res. Commun. **188** 636-642.

White, D.R., and Aitken, R.J. (1989). relationship between calcium, cyclic AMp, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. Gamete Research **22** 163-177.

Yanagimachi, R. (1970). The movement of golden hamster spermatozoa before and after capacitation. J. Reprod. Fert. **23** 193-196.

Yanagimachi, R. (1981). Mechanism of fertilization in mammals. Fertilization and Embryonic Development in vitro (Mastroianni, L., and Biggers, J.D., eds.) Plenum Press, N. York 81-182.

Yanagimachi, R. (1988). Mammalian Fertilization. In: The physiology of reproduction (Knobil and Neill eds.) Ravan Press. 135-185.

Young, L.G., and Nelson, L. (1974). The effects of heavy metal ions on the motility of sea urchin spermatozoa. Biol. Bull. **147** 236-246.

RAW DATA

RAW DATA

I. MAMMALIAN SPERMATOZOA

Table One - The Effect Of SITS On The Motility Of Sperm

Conc of SITS	% MOTILITY		Conc of SITS	% MOTILITY	
	CAPUT	CAUDAL		CAPUT	CAUDAL
Control	47	80	0.1 mM	38	69
	47	73		40	72
	43	81		40	70
	48	82		40	68
	42	76		42	68
	44	75		42	72
0.5 mM	36	55	1 mM	19	22
	33	51		19	22
	33	53		16	22
	34	49		18	26
	32	50		17	21
	30	53		18	22

Table Two - The Effect Of Monensin On The Motility Of Sperm

Conc of Monensin	% MOTILITY		Conc of Monensin	% MOTILITY	
	CAPUT	CAUDAL		CAPUT	CAUDAL
0	38	74	0.1	50	82
	38	80		55	78
	36	81		52	80
	39	73		51	80
	35	74		54	79
	36	80		52	83
1	68	86	10	91	92
	69	83		84	99
	63	85		94	99
	65	91		87	90
	68	90		87	98
	64	92		91	92

Table Three - The Effect Of Theophylline On The Motility Of Sperm

Condition	% MOTILITY		Condition	% MOTILITY	
	CAPUT	CAUDAL		CAPUT	CAUDAL
Control	0	24	+Bicarbonate	41	88
	0	28		50	87
	4	31		40	83
	2	23		49	88
	4	25		46	82
	1	32		42	90
	3	28		41	86
	5	32		48	80
	4	32		44	84
	1	24		49	82
+Theophylline	15	88	+Bicarbonate &	64	91
	16	86	+Theophylline	62	82
	13	82		75	83
	4	87		66	81
	12	83		70	89
	10	81		71	85
	6	81		65	92
	18	85		72	90
	7	85		74	84
	9	83		62	93

Table Four - The Effect Of Forskolin On The Motility Of Sperm

Conc Of Forskolin	% MOTILITY CAPUT -Bicarbonate	+Bicarbonate	% MOTILITY CAUDAL -Bicarbonate	+Bicarbonate
Control	5	48	29	82
	5	46	27	84
	4	45	26	78
	1	42	35	78
	1	42	30	76
	3	45	25	82
0.1	5	60	30	86
	4	59	30	87
	1	58	30	87
	4	61	30	89
	2	57	29	90
	3	56	31	89
1	3	63	36	96
	3	69	35	99
	4	65	35	96
	3	64	38	96
	3	67	34	96
	3	68	35	96
10	5	78	42	100
	4	79	44	99
	4	72	40	100
	4	77	38	99
	4	78	39	99
	4	84	41	99

Table Five - The Effect Of Verapamil On The Motility Of Sperm

Conc Of Verapamil	% MOTILITY CAPUT -Bicarbonate	+Bicarbonate	% MOTILITY CAUDAL -Bicarbonate	+Bicarbonate
0	0	40	28	81
	2	42	30	88
	2	43	25	87
	4	43	26	82
	2	40	24	83
	4	44	30	89
0.1	0	29	19	76
	0	31	19	78
	0	31	19	74
	0	30	22	74
	0	32	21	76
	0	33	23	78
10	0	24	22	65
	0	22	16	68
	0	28	16	66
	0	24	15	69
	0	26	16	69
	0	26	16	65
100	0	17	7	57
	0	17	9	56
	0	21	11	56
	0	21	13	54
	0	13	10	53
	0	13	10	54

Table Six - The Effect Of PMA On The Motility Of Sperm

Conc Of PMA	% MOTILITY CAPUT -Bicarbonate	+Bicarbonate	% MOTILITY CAUDAL -Bicarbonate	+Bicarbonate
0	0	37	30	47
	2	38	25	48
	2	43	24	48
	3	42	29	43
	2	42	30	45
	4	41	25	42
1	4	48	32	67
	4	51	29	68
	2	50	28	64
	6	47	28	65
	3	48	32	68
	2	50	31	64
10	5	57	37	77
	5	55	35	79
	3	61	41	81
	4	59	39	84
	4	61	37	83
	3	55	42	76
100	4	82	42	100
	4	74	43	96
	5	74	40	99
	2	78	48	100
	3	78	45	100
	6	82	46	96

Table Seven - The Effect Of SITS On PMA - Stimulated Motility

	% MOTILITY		
Control	+ PMA	+Bicarbonate +PMA	+Bicarbonate +PMA + SITS
28	44	100	34
24	45	100	32
24	41	100	28
32	42	96	26
28	44	100	35
32	42	95	35

Table Eight - The Effect Of H.7 On The Motility Of Sperm

	% MOTILITY
Control	+ H-7
90	23
88	23
92	14
86	19
82	25
84	18

11. *FUCUS SERRATUS*

Although readings from linear traces were made at time periods varying between 5 - 15 minutes the results are resented as the number of divisions / min for convenience. Each division represents 11.75 nmoles O_2/ min (see materials and methods section)

Table One - The Effect Of pH On The Motility And Oxygen Uptake Of Sperm

Absorbance at 340nm	No Of Sperm x 10^3	% Motility	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^3	% Motility	No Divisions / min
pH5				pH6			
0.377	1.2	20	1.512	0.345	1.1	30	1.13
0.424	1.35	22	1.755	0.314	1.0	25	1.045
0.345	1.1	14	1.339	0.314	1.0	33	0.722
0.377	1.2	18	1.135	0.659	2.1	35	1.508
0.440	1.4	16	1.774	0.597	1.9	34	1.663
0.471	1.5	24	1.424	0471.	1.5	27	1.560
0.408	1.3	21	1.625	0.314	1.0	26	1.015
0.455	1.45	12	1.599	0.345	1.1	22	0.792
0.471	1.5	19	1.359	0.377	1.2	31	0.876
0.408	1.3	20	1.286	0.440	1.4	38	0.987
0.314	1.0	16	1.253	0.314	1.0	37	1.032
0.345	1.1	13	1.048	0.345	1.1	34	0.781
0.408	1.3	23	1.221	0.345	1.1	29	1.131
0.408	1.3	17	1.235	0.349	1.2	23	1.224
0.314	1.0	15	1.263	0.377	1.2	26	0.882
pH7				pH8			
0.565	1.8	51	5.116	0.314	1.0	85	4.26
0.314	1.0	48	2.834	0.345	1.1	78	4.044
0.440	1.4	49	2.990	0.471	1.5	92	5.577
0.455	1.45	54	3.109	0.471	1.5	95	5.451
0.471	1.5	53	3.132	0.471	1.5	80	6.425
0.502	1.6	45	3.507	0.471	1.5	90	5.480
0.440	1.4	57	2.969	0.565	1.8	75	7.609
0.408	1.3	53	2.766	0.550	1.75	89	7.525
0.408	1.3	49	3.675	0.502	1.6	91	6.675
0.408	1.3	51	2.796	0.314	1.0	81	3.764
0.393	1.25	52	3.475	0.345	1.1	81	3.937
0.502	1.6	51	4.571	0.377	1.2	79	4.420
0.534	1.7	51	4.845	0.377	1.2	94	4.762
0.597	1.9	51	4.729	0.314	1.0	76	4.253
0.314	1.0	50	2.890	0.345	1.1	89	4.350
pH9				pH10			
0.314	1.0	54	2.25	0.345	1.1	39	1.144
0.345	1.1	60	2.024	0.314	1.0	46	1.060
0.283	0.9	50	1.670	0.314	1.0	34	1.740
0.345	1.1	48	2.527	0.408	1.3	40	2.159
0.408	1.3	58	2.659	0.440	1.4	50	1.595
0.471	1.5	59	3.305	0.502	1.6	39	2.389
0.628	2.0	55	3.648	0.534	1.7	28	2.222
0.345	1.1	51	2.041	0.534	1.7	52	2.083
0.408	1.3	49	2.919	0.597	1.9	41	2.822
0.377	1.2	57	2.694	0.345	1.1	36	1.988
0.314	1.0	53	1.793	0.314	1.0	45	1.00
0.330	1.05	47	2.379	0.377	1.2	37	2.112
0.534	1.7	52	3.798	0.345	1.1	35	1.727
0.314	1.0	61	1.887	0.314	1.0	40	1.23
0.377	1.2	56	2.202	0.314	1.0	40	1.48

Table Two - The Effect Of Temperature On Sperm Motility

The concentration of sperm was adjusted to 1 x 10^8 sperm/ml in all experiments

Temperature oC	% Motility	Temperature oC	% Motility	Temperature oC	% Motility
4	10	6	21	10	85
	6		23		80
	10		22		87
	8		22		83
	8		24		90
	7		17		81
	9		20		89
	11		23		79
	5		18		91
	8		20		96
	6		22		83
	6		19		87
	10		25		74
	7		20		78
	9		19		92
25	50	37	23		
	52		20		
	58		27		
	47		19		
	40		22		
	48		25		
	42		25		
	46		21		
	49		26		
	60		24		
	51		24		
	54		21		
	52		22		
	48		25		
	53		21		

Table Three - The Effect Of The Ionic Composition Of ASW On The Motility And Oxygen Uptake Of Spermatozoa

Abeorbance at 340nm	No Of Sperm x 10^7	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^7	% Motility	No Divisions / min
Control				-Na+			
0.314	1.0	80	1.577	0.565	1.8	1	1.606
0.345	1.1	88	1.76	0.534	1.7	0.5	0.830
0.345	1.1	78	1.619	0.534	1.7	0.5	1.309
0.408	1.3	90	1.914	0.345	1.1	1	0.671
0.298	0.95	83	1.598	0.314	1.0	1	0.902
0.377	1.2	80	1.904	0.377	1.2	1	0.574
0.377	1.2	85	1.430	0.408	1.3	1	0.637
0.597	1.9	88	3.034	0.565	1.8	0.5	1.602
0.408	1.3	86	1.884	0.597	1.9	1	1.663
0.440	1.4	82	2.085	0.722	2.3	0.5	1.162
-K+				-Ca 2+			
0.440	1.4	82	5.421	0.345	1.1	27	1.606
0.345	1.1	72	4.250	0.408	1.3	22	1.544
0.345	1.1	69	4.261	0.283	0.9	26	1.105
0.314	1.0	85	3.496	0.471	1.5	28	2.265
0.283	0.9	80	3.154	0.502	1.6	23	1.789
0.314	1.0	74	3.495	0.565	1.8	25	2.610
0.408	1.3	79	5.101	0.597	1.9	24	2.789
0.440	1.4	81	4.822	0.345	1.1	24	1.326
0.534	1.7	75	6.433	0.314	1.0	26	1.451
0.283	0.9	73	3.226	0.314	1.0	25	1.206
-Mg 2+							
0.534	1.7	31	2.681				
0.565	1.8	27	2.857				
0.471	1.5	29	2.396				
0.471	1.5	29	2.400				
0.440	1.4	30	2.061				
0.502	1.6	33	1.907				
0.471	1.5	28	2.231				
0.314	1.0	29	1.472				
0.345	1.1	25	1.850				
0.377	1.2	29	1.787				

Table Four - The Effect Of Extracellular pH On Coupled And Uncoupled Respiration

Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min
-FCCP					
pH5			pH6		
0.597	1.9	0.874	0.377	1.2	1.828
0.659	2.1	0.861	0.408	1.3	1.840
0.785	2.5	0.75	0.408	1.3	1.411
0.314	1.0	0.26	0.377	1.2	1.320
0.440	1.4	0.658	0.377	1.2	1.860
0.345	1.1	0.264	0.345	1.1	1.075
pH7			pH8		
0.471	1.5	3.735	0.722	2.3	9.005
0.377	1.2	3.702	0.314	1.0	3.245
0.377	1.2	3.612	0.314	1.0	3.235
0.408	1.3	3.270	0.597	1.9	7.467
0.440	1.4	3.612	0.628	2.0	6.520
0.597	1.9	5.928	0.565	1.8	6.957
+FCCP					
pH5			pH6		
0.440	1.4	0.886		1.0	1.578
0.408	1.3	0.844		1.0	1.269
0.408	1.3	0.477		1.0	1.343
0.393	1.25	0.464		1.9	2.736
0.471	1.5	0.942		1.0	1.200
0.345	1.1	0.387		1.1	1.690
pH7			pH8		
0.597	1.9	6.128	0.345	1.1	4.326
0.565	1.8	5.751	0.345	1.1	3.539
0.345	1.1	2.921	0.377	1.2	3.738
0.267	0.85	2.690	0.377	1.2	4.850
0.345	1.1	2.987	0.314	1.0	3.455
0.408	1.3	3.491	0.283	0.9	3.319

Table Five - The Effect Of Na+ On Oxygen Uptake

Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min
Control			+360mM Na+		
0.597	1.9	7.315	0.251	0.8	1.109
0.722	2.3	8.211	0.440	1.4	2.017
0.534	1.7	6.584	0.440	1.4	1.268
0.314	1.0	3.717	0.565	1.8	1.667
0.565	1.8	6.665	0.565	1.8	1.566
0.565	1.8	6.385	0.440	1.4	1.890
0.283	0.9	3.180	0.471	1.5	1.373
0.345	1.1	3.968	0.502	1.6	1.304
0.314	1.0	3.887	0.471	1.5	2.012
0.408	1.3	4.957	0.471	1.5	1.995

Table Six - The Effect Of N-hexane On The Motility And Oxygen Uptake Of Sperm

Absorbance at 340nm	No Of Sperm x 10^{8}	% Motility	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^{Y}	% Motility	No Divisions / min
Control				±1mM hexane			
0.408	1.3	84	5.272	0.440	1.4	99	16.407
0.565	1.8	86	6.561	0.408	1.3	99	15.133
0.785	2.5	83	9.843	0.408	1.3	99	14.859
0.314	1.0	83	3.763	0.377	1.2	99	13.914
0.345	1.1	85	3.990	0.345	1.1	98	13.123
0.377	1.2	87	4.888	0.314	1.0	99	11.772
0.408	1.3	84	5.369	0.440	1.4	99	16.636
0.440	1.4	86	5.544	0.345	1.1	99	12.606
0.314	1.0	87	3.570	0.314	1.0	99	11.468
0.345	1.1	85	4.114	0.314	1.0	99	11.898
± 10mM hexane							
0.283	0.9	94	6.704				
0.345	1.1	94	8.245				
0.345	1.1	94	7.960				
0.314	1.0	93	7.179				
0.408	1.3	94	9.610				
0.816	2.6	94	18.980				
0.314	1.0	95	7.472				
0.314	1.0	94	7.133				
0.345	1.1	94	8.061				
0.345	1.1	94	7.872				

Table Seven - The Effect Of Na+ On The Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10⁶	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10⁶	No Divisions / min
±50mM			±100mM		
0.345	1.1	0.627	0.314	1.0	1.277
0.377	1.2	0.504	0.314	1.0	1.151
0.408	1.3	0.507	0.314	1.0	1.304
0.440	1.4	0.770	0.314	1.0	1.124
0.440	1.0	0.560	0.314	1.0	1.187
0.314	1.1	0.574	0.314	1.0	1.099
0.345	1.1	0.226	0.314	1.0	1.241
0.345	1.1	0.831	0.314	1.0	1.329
0.377	1.2	0.600	0.314	1.0	1.225
0.345	1.1	0.473	0.314	1.0	1.203
±200mM			±300mM		
0.314	1.0	2.430	0.408	1.3	4.355
0.314	1.0	2.256	0.471	1.5	4.283
0.251	0.8	1.840	0.659	2.1	7.350
0.471	1.5	3.582	0.345	1.1	2.970
0.628	2.0	4.610	0.283	0.9	2.700
0.345	1.1	2.621	0.314	1.0	3.410
0.408	1.3	3.250	0.314	1.0	3.210
0.345	1.1	2.409	0.440	1.4	3.914
0.471	1.5	3.375	0.440	1.4	4.424
0.440	1.4	3.413	0.440	1.4	4.264
±360mM			±400mM		
0.345	1.1	4.092	0.314	1.0	3.590
0.597	1.9	6.574	0.345	1.1	3.256
0.314	1.0	3.850	0.534	1.7	5.780
0.283	0.9	2.970	0.722	2.3	7.245
0.408	1.3	4.459	0.816	2.6	8.840
0.408	1.3	4.875	0.314	1.0	2.950
0.408	1.3	4.550	0.314	1.0	3.580
0.408	1.3	4.654	0.283	0.9	2.853
0.408	1.3	4.680	0.314	1.0	2.800
0.345	1.1	4.048	0.408	1.3	4.875
±500mM					
0.314	1.0	2.000			
0.314	1.0	1.980			
0.314	1.0	1.990			
0.314	1.0	1.884			
0.314	1.0	1.900			
0.314	1.0	2.000			
0.314	1.0	2.100			
0.314	1.0	1.960			
0.314	1.0	2.080			
0.314	1.0	2.025			

Table Eight - The Effect Of Amiloride On The Motility And Oxygen Uptake Of Sperm

Absorbance at 340nm	No Of Sperm x 10^3	% Motility	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^3	% Motility	No Divisions / min
Control				±1mM			
0.408	1.3	80	4.581	0.879	2.8	74	10.335
0.345	1.1	86	4.517	0.659	2.1	70	6.403
0.314	1.0	78	4.033	1.005	3.2	77	10.864
0.345	1.1	88	4.012	0.314	1.0	66	3.345
0.377	1.2	91	4.753	0.440	1.4	69	4.829
0.377	1.2	89	4.535	0.534	1.7	73	5.459
0.534	1.7	85	6.807	0.691	2.2	67	7.128
0.597	1.9	81	7.078	0.408	1.3	70	4.251
0.628	2.0	75	7.550	0.440	1.4	70	4.900
0.722	2.3	77	8.924	0.314	1.0	66	3.470
0.314	1.0	88	-	0.314	1.0	74	-
0.314	1.0	83	-	0.314	1.0	71	-
0.283	0.9	78	-	0.314	1.0	63	-
0.345	1.1	76	-	0.345	1.1	66	-
0.315	1.0	90	-	0.440	1.4	74	-
±10mM				± 50mM			
0.377	1.2	51	3.001	0.345	1.1	30	1.579
0.345	1.1	56	2.738	0.377	1.2	32	1.698
0.345	1.1	50	2.607	0.267	0.85	22	1.160
0.314	1.0	41	2.350	0.345	1.1	27	1.480
0.377	1.2	48	3.001	0.440	1.4	32	1.932
0.440	1.4	54	3.506	0.377	1.2	31	1.680
0.597	1.9	47	4.674	0.377	1.2	30	1.770
0.534	1.7	42	4.175	0.283	0.9	24	1.175
0.408	1.3	56	3.175	0.565	1.8	27	2.443
0.408	1.3	42	3.163	0.345	1.1	29	1.565
0.314	1.0	49	-	0.345	1.1	25	-
0.298	0.95	47	-	0.345	1.1	23	-
0.314	1.0	51	-	0.314	1.0	27	-
0.314	1.0	57	-	0.283	0.9	22	-
0.314	1.0	44	-	0.314	1.0	24	-
±100mM							
0.502	1.6	36	2.966				
0.565	1.8	34	3.040				
0.502	1.6	38	2.885				
0.502	1.6	37	2.878				
0.345	1.1	32	2.203				
0.267	0.85	35	1.503				
0.754	2.4	31	4.272				
0.314	1.0	33	1.802				
0.628	2.0	36	3.611				
0.534	1.7	32	3.041				
0.471	1.5	34	-				
0.440	1.4	30	-				
0.345	1.1	34	-				
0.345	1.1	32	-				
0.314	1.0	36	-				

Table Nine - The Effect Of Monensin On The Oxygen Uptake Of Sperm

Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min
Control			±0.01mM		
0.502	1.6	6.722	0.345	1.1	6.721
0.534	1.7	7.161	0.565	1.8	9.828
0.565	1.8	7.308	0.565	1.8	9.679
0.345	1.1	4.400	0.314	1.0	6.193
0.314	1.0	3.910	0.314	1.0	6.030
0.377	1.2	4.758	0.597	1.9	10.526
0.534	1.7	6.741	0.659	2.1	11.529
0.534	1.7	6.809	0.879	2.8	15.218
0.565	1.8	7.794	0.314	1.0	6.130
0.754	2.4	8.736	0.283	0.9	5.477
±0.05mM			±0.1mM		
0.345	1.1	18.667	0.471	1.5	34.061
0.502	1.6	25.712	0.879	2.8	60.824
0.581	1.85	31.580	0.534	1.7	37.014
0.597	1.9	30.723	0.597	1.9	43.052
0.659	2.1	33.804	0.502	1.6	36.184
0.879	2.8	47.720	0.377	1.2	26.178
0.408	1.3	22.075	0.345	1.1	24.923
0.345	1.1	17.775	0.345	1.1	23.950
0.314	1.0	16.680	0.314	1.0	22.757
0.471	1.5	24.690	0.283	0.9	19.506
0.5mM			±1mM		
0.314	1.0	13.772	0.502	1.6	11.142
0.314	1.0	11.818	0.408	1.3	7.795
0.314	1.0	11.734	0.345	1.1	6.614
0.314	1.0	11.902	0.283	0.9	6.252
0.314	1.0	13.856	0.754	2.4	16.762
0.314	1.0	13.895	0.879	2.8	19.312
0.314	1.0	13.688	0.597	1.9	11.520
0.314	1.0	13.649	0..534	1.7	11.822
0.314	1.0	11.695	0.377	1.2	8.437
0.314	1.0	11.941	0.314	1.0	5.929

Table Ten - The Effect Of Monensin And Amiloride On The Oxygen Uptake Of Sperm Incubated In ONaASW

Absorbance at 340nm	No Of Sperm x 10^3	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^3	No Divisions / min
ONaASW			+Monensin		
0.440	1.4	1.211	0.345	1.1	0.674
0.440	1.4	1.124	0.314	1.0	0.569
0.345	1.1	0.561	0.314	1.0	0.725
0.314	1.0	0.751	0.314	1.0	0.769
0.283	0.9	0.564	0.345	1.1	0.908
0.377	1.2	0.689	0.314	1.0	0.513
0.377	1.2	0.989	0.565	1.8	0.990
0.440	1.4	1.162	0.659	2.1	1.142
0.345	1.1	0.603	0.754	2.4	1.891
0.314	1.0	0.554	0.754	2.4	1.906
+Amiloride					
0.785	2.5	0.458			
0.565	1.8	0.211			
0.565	1.8	0.387			
0.785	2.5	0.213			
0.314	1.0	0.182			
0.345	1.1	0.130			
0.314	1.0	0.191			
0.314	1.0	0.091			
0.283	0.9	0.185			
0.565	1.8	0.202			

Table Eleven - The Effect Of Ouabain On The Motility And Oxygen Uptake Of Sperm

Absorbance at 340nm	No Of Sperm x 10^3	% Motility	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^3	% Motility	No Divisions / min
Control				+0.01mM			
0.314	1.0	89	4.751	0.534	1.7	50	4.464
0.314	1.0	71	4.597	0.565	1.8	62	4.687
0.408	1.3	68	5.988	0.471	1.5	48	5.000
0.534	1.7	92	7.871	0.534	1.7	58	4.712
0.659	2.1	91	10.164	0.534	1.7	64	5.726
0.471	1.5	82	6.573	0.471	1.5	55	4.839
0.471	1.5	77	7.617	0.283	0.9	57	3.066
0.440	1.4	69	6.275	0.754	2.4	70	6.113
0.408	1.3	83	6.214	0.314	1.0	42	3.367
0.471	1.5	82	6.569	0.345	1.1	54	2.866
+0.1mM				+1mM			
0.565	1.8	24	3.019	0.597	1.9	38	5.113
0.440	1.4	27	2.528	0.314	1.0	26	2.122
0.408	1.3	17	2.551	0.345	1.1	29	2.716
0.377	1.2	22	2.004	0.440	1.4	41	3.571
0.502	1.6	20	2.702	0.471	1.5	35	3.828
0.502	1.6	25	2.878	0.408	1.3	37	3.011
0.565	1.8	19	3.008	0.754	2.4	23	5.436
0.659	2.1	22	4.364	0.628	2.0	28	4.246
0.408	1.3	23	2.401	0.471	1.5	27	3.284
0.377	1.2	21	2.353	0.440	1.4	36	2.930
+10mM							
0.440	1.4	17	1.025				
0.502	1.6	15	0.840				
0.471	1.5	15	0.906				
0.345	1.1	13	0.904				-
0.314	1.0	16	0.852				-
0.267	0.85	19	0.675				-
0.471	1.5	14	1.023				-
0.534	1.7	15	1.396				-
0.597	1.9	15	1.096				
0.628	2.0	11	1.152				

Table Twelve- The Effect Of Lithium On The Motility And Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min
Control				LiASW			
0.597	1.9	89	7.942	0.471	1.5	26	2.385
0.345	1.1	90	4.637	0.471	1.5	25	3.375
0.408	1.3	87	5.174	0.565	1.8	19	3.834
0.408	1.3	88	5.142	0.314	1.0	18	1.680
0.345	1.1	91	4.235	0.345	1.1	20	1.755
0.345	1.1	95	4.455	0.471	1.5	24	3.323
0.314	1.0	88	4.239	0.502	1.6	21	2.614
0.283	0.9	90	3.434	0.408	1.3	15	2.093
0.440	1.4	83	5.307	0.314	1.0	29	2.200
0..377	1.2	89	4.890	0.314	1.0	23	2.176

Table Thirteen - The Effect Of Ouabain On The Oxygen Uptake Of Sperm Incubated In ONaASW

Abeorbance at 340nm	No Of Sperm x 10^8	No Divisions / min
+Ouabain		
0.314	1.0	0.176
0.345	1.1	0.216
0.722	2.3	0.380
0.471	1.5	0.141
0.408	1.3	0.267
0.345	1.1	0.081
0.283	0.9	0.095
0.440	1.4	0.203
0.534	1.7	0.213
0.565	1.8	0.117

Table Fourteen- The Effect Of SITS On The Motility And Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min
Control				±10μM			
0.345	1.1	85	3.611	0.314	1.0	30	1.342
0.408	1.3	85	4.163	0.754	2.4	29	3.667
0.408	1.3	83	3.752	0.565	1.8	32	2.635
0.440	1.4	87	4.703	0.659	2.1	30	2.993
0.785	2.5	84	8.553	0.628	2.0	30	2.792
0.628	2.0	87	6.916	0.597	1.9	30	2.375
0.942	3.0	83	9.156	0.471	1.5	32	1.928
0.314	1.0	84	3.377	0.471	1.5	29	2.100
0.267	0.85	86	2.927	0.471	1.5	31	2.003
0.408	1.3	82	3.986	0.471	1.5	30	2.367
0.345	1.1	88	3.581	0.345	1.1	30	1.564
0.345	1.1	85	3.851	0.440	1.4	30	2.185
0.314	1.0	87	3.069	0.345	1.1	30	1.608
0.440	1.4	83	4.213	0.345	1.1	28	1.697
0.408	1.3	86	4.473	0.565	1.8	31	2.743
±100μM				±500μM			
0.314	1.0	23	0.962	0.345	1.1	40	1.388
0.314	1.0	23	0.938	0.345	1.1	37	1.471
0.236	0.75	23	0.772	0.659	2.1	39	2.545
0.722	2.3	23	2.139	0.565	1.8	36	2.500
0.848	2.7	23	2.703	0.314	1.0	38	1.260
0.659	2.1	25	2.075	0.314	1.0	42	1.170
0.942	3.0	21	2.985	0.267	0.85	40	1.137
0.314	1.0	23	0.983	0.471	1.5	38	2.00
0.440	1.4	23	1.326	0.47	1.5	39	1.835
0.471	1.5	24	1.532	0.565	1.8	37	2.117
0.502	1.6	24	1.763	0.267	0.85	39	1.193
0.502	1.6	22	1.709	0.314	1.0	36	1.068
0.502	1.6	25	1.578	0.408	1.3	38	1.413
0.565	1.8	23	1.786	0.440	1.4	34	1.632
				0.534	1.7	36	1.885
±1000μM							
0.377	1.2	49	1.886				
0.565	1.8	45	2.768				
0.408	1.3	46	1.905				
0.345	1.1	50	1.851				
0.251	0.8	48	1.345				
0.471	1.5	48	1.916				
0.471	1.5	44	2.400				
0.471	1.5	45	2.679				
0.722	2.3	45	4.078				
0.565	1.8	49	2.727				
0.597	1.9	46	2.918				
0.659	2.1	47	3.200				
0.440	1.4	49	1.939				
0.377	1.2	45	2.143				
0.597	1.9	48	3.247				

Table Fifteen - The Effect Of Verapamil On The Motility And Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^5	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^5	% Motility	No Divisions / min
Control				±0.1µM			
0.440	1.4	89	5.922	0.565	1.8	43	3.173
0.471	1.5	86	5.490	0.597	1.9	43	3.186
0.471	1.5	92	6.195	0.848	2.7	43	6.923
0.471	1.5	91	5.790	0.314	1.0	41	1.464
0.471	1.5	85	5.640	0.267	0.85	45	1.211
0.565	1.8	87	7.254	0.440	1.4	40	2.426
0.785	2.5	88	10.825	0.408	1.3	43	2.743
0.314	1.0	93	3.560	0.408	1.3	46	3.333
0.440	1.4	93	5.537	0.345	1.1	42	2.00
0.408	1.3	88	5.116	0.314	1.0	43	1.677
0.314	1.0	84	4.230	0.314	1.0	42	1.836
0.314	1.0	89	3.660	0.298	0.95	44	1.397
0.471	1.5	90	6.045	0.534	1.7	43	1.974
0.471	1.5	85	5.790	0.565	1.8	44	3.375
0.502	1.6	94	6.312	0.659	2.1	43	2.457
±1µM				±10µM			
0.471	1.5	40	1.203	0.597	1.9	38	2.366
0.471	1.5	38	1.445	0.471	1.5	36	1.616
0.471	1.5	40	1.418	0.440	1.4	36	1.448
0.314	1.0	40	0.938	0.345	1.1	39	2.222
0.345	1.1	39	0.776	0.345	1.1	35	0.961
0.345	1.1	40	0.681	0.314	1.0	37	0.856
0.408	1.3	39	0.801	0.377	1.2	36	0.936
0.440	1.4	39	1.561	0.440	1.4	36	2.043
0.534	1.7	39	0.365	0.597	1.9	36	3.528
0.565	1.8	39	1.100	0.879	2.8	34	2.528
0.597	1.9	38	1.362	0.502	1.6	37	2.122
0.471	1.5	39	1.452	0.471	1.5	32	1.670
0.471	1.5	37	1.385	0.440	1.4	39	1.814
0.345	1.1	38	1.084	0.408	.3	35	1.174
0.314	1.0	39	0.657	0.377	1.2	33	1.926
±100µM				±500µM			
0.314	1.0	28	0.777	0.330	1.05	23	1.067
0.314	1.0	32	1.290	0.345	1.1	25	0.796
0.345	1.1	26	1.022	0.408	1.3	20	0.846
0.377	1.2	24	1.600	0.565	1.8	26	0.881
0.534	1.7	27	1.981	0.534	1.7	21	0.793
0.440	1.4	21	1.093	0.471	1.5	20	0.764
0.408	1.3	24	1.151	0.471	1.5	20	0.813
0.440	1.4	29	3.059	0.440	1.4	25	0.976
0.440	1.4	35	1.757	0.345	1.1	21	1.130
0.471	1.5	31	1.199	0.345	1.1	22	0.980
0.502	1.6	22	2.488	0.314	1.0	26	0.985
0.534	1.7	30	1.057	0.597	1.9	24	1.198
0.345	1.1	30	1.222	0.659	2.1	22	1.081
0.314	1.0	28	0.748	0.628	2.0	26	0.709
0.345	1.1	34	1.241	0.471	1.5	25	0.634
±1000µM							
0.597	1.9	14	1.412				
0.597	1.9	16	1.647				
0.659	2.1	15	1.149				
0.471	1.5	12	0.750				
0.502	1.6	18	0.770				
0.532	1.7	11	1.023				
0.597	1.9	13	1.315				
0.345	1.1	17	0.677				
0.377	1.2	14	0.630				
0.345	1.1	14	0.541				
0.377	1.2	14	1.021				
0.377	1.2	10	0.650				
0.440	1.4	15	0.774				
0.471	1.5	13	0.995				
0.471	1.5	14	0.813				

Table Sixteen - The Uptake And Hydrolysis Of BCECF - AM

Time (Min)	Fluorescence Readings (excitation = 465nm) Control	Heat Treated (80°c)	Triton x 100 - Treated
0	12.26	15.20	8.30
	12.40	14.97	9.00
	12.16	13.12	7.98
	12.30	15.00	8.50
	12.27	13..32	7.78
	12.29	13.33	
15	22.90	17.55	17.90
	22.89	17.00	17.42
	23.08	18.25	18.00
	23.06	14.95	17.31
	22.92	15.50	17.82
	23.09	14.24	17.50
30	48.90	19.60	34.03
	48.56	17.56	34.60
	47.66	17.69	33.02
	49.80	19.47	33.60
	48.75	19.33	32.46
	48.71	17.83	33.45
45	93.90	24.60	51.97
	92.82	25.10	50.67
	87.68	22.50	50.61
	89.76	22.00	53.00
	91.80	23.10	49.64
	89.76	24.00	52.03
60	158.50	30.67	78.00
	155.10	31.07	77.53
	154.90	29.68	76.01
	155.69	29.27	76.46
	158.30	30.28	75.50
	157.70	30.06	78.50
75	241.90	45.60	113.30
	232.30	45.80	110.29
	235.09	45.00	114.81
	239.89	44.79	112.00
	239.11	46.00	108.80
	234.31	44.60	111.60
90	309.21	62.05	135.50
	308.70	58.85	134.92
	307.70	58.35	133.80
	307.19	62.55	131.48
	308.81	59.96	132.60
	307.60	60.95	130.90
105	392.50	81.49	160.40
	391.30	77.89	162.00
	388.51	78.78	161.43
	390.50	82.70	170.00
	387.80	80.60	160.80
	386.49	76.68	159.77
120	460.09	105.50	193.45
	466.11	104.00	191.36
	463.15	104.50	192.00
	461.05	103.50	195.70
	465.15	105.00	189.10
	463.06	104.50	192.81

Table Seventeen - The Effect Of BCECF - AM On The Motility And Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^6	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^6	% Motility	No Divisions / min
Control							
0h				0.5h			
0.157	0.5	93	2.050	0.314	1.0	82	4.000
0.283	0.9	90	3.600	0.408	1.3	86	5.512
0.267	0.85	90	3.175	0.408	1.3	85	5.096
0.157	0.5	87	2.080	0.502	1.6	85	6.712
0.597	1.9	92	7.410	0.534	1.7	84	6.248
0.345	1.1	89	4.147	0.597	1.9	83	7.030
1h				1.5h			
0.565	1.8	87	7.380	0.314	1.0	86	3.690
0.659	2.1	91	7.875	0.345	1.1	84	3.949
0.534	1.7	92	6.715	0.534	1.7	84	6.707
0.565	1.8	90	7.470	0.659	2.1	85	8.400
0.597	1.9	86	7.030	0.471	1.5	87	6.150
0.659	2.1	88	7.980	0.471	1.5	83	5.618
2h				2.5h			
0.471	1.5	80	5.573	0.314	1.0	70	3.462
0.471	1.5	81	5.760	0.345	1.1	58	4.413
0.471	1.5	79	5.798	0.408	1.3	67	5.155
0.471	1.5	74	5.727	0.534	1.7	72	7.075
0.534	1.7	73	6.358	0.754	2.4	56	8.667
0.565	1.8	75	6.772	0.314	1.0	61	3.463
3h				3.5h			
0.157	0.5	54	2.006	0.314	1.0	53	3.515
0.659	2.1	62	8.652	0.314	1.0	47	3.590
0.754	2.4	56	8.916	0.314	1.0	54	3.525
0.408	1.3	53	4.685	0.314	1.0	55	3.495
0.534	1.7	63	6.140	0.314	1.0	46	3.565
0.565	1.8	60	6.496	0.314	1.0	50	3.575
4h							
0.597	1.9	46	6.080				
0.314	1.0	44	3.215				
0.157	0.5	47	1.518				
0.345	1.1	38	3.410				
0.377	1.2	40	3.798				
0.565	1.8	38	5.553				

Table 17 Continued .....

+BCECF -AM

Absorbance at 340nm	No Of Sperm x 10^{8}	% Motility	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^{8}	% Motility	No Divisions / min
0h				0.5h			
0.345	1.1	89	4.313	0.314	1.0	86	4.017
0.565	1.8	91	6.392	0.314	1.0	74	3.919
0.597	1.9	85	6.690	0.597	1.9	83	6.880
0.754	2.4	87	7.478	0.408	1.3	88	5.350
0.408	1.3	83	5.061	0.440	1.4	72	5.215
0.408	1.3	86	4.653	0.471	1.5	77	5.294
1h				1.5h			
0.534	1.7	87	7.339	0.659	2.1	79	9.345
0.440	1.4	85	6.076	0.314	1.0	80	3.950
0.565	1.8	77	7.115	0.267	0.85	73	3.316
0.597	1.9.	80	7.505	0.440	1.4	72	6.161
0.408	1.3	74	5.646	0.502	1.6	75	7.198
0.345	1.1	75	4.342	0.565	1.8	78	7.198
2h				2.5h			
0.565	1.8	79	7.186	0.267	0.85	66	3.228
0.754	2.4	63	9.197	0314	1.0	68	3.498
0.973	3.1	66	12.564	0.471	1.5	63	5.724
0.157	0.5	81	1.947	0.502	1.6	62	5.568
0.440	1.4	60	5.503	0.345	1.1	55	3.868
0.345	1.1	76	4.148	0.408	1.3	58	4.914
3h				3.5h			
0.314	1.0	53	3.780	0.314	1.0	45	3.830
0.408	1.3	47	4.745	0.314	1.0	44	3.370
0.440	1.4	46	5.074	0.314	1.0	42	3.415
0.597	1.9	55	7.133	0.314	1.0	43	3.785
0.659	2.1	51	7.720	0.314	1.0	41	3.875
0.471	1.5	49	5.709	0.314	1.0	44	3.325
4h							
0.283	0.9	47	3.330				
0.345	1.1	39	3.080				
0.597	1.9	37	5.419				
0.754	2.4	48	8.755				
0.440	1.4	43	3.847				
0.565	1.8	44	6.754				

Table Eighteen - The Effect Of The Composition Of ASW On The Intracellular pH Of Sperm

Extracellular pH	Fluorescence Readings 495nm	450nm	Fluorescence Readings After Digitonin 495nm	450nm
ONaASW				
6.0	180	38	198	55
	199	48	238	78
	167	47	165	69
6.4	205	74	235	111
	279	109	315	180
	282	111	306	193
6.8	144	27	188	39
	112	27	173	47
	163	29	209	40
7.2	299	91	386	125
	276	66	401	99
	322	147	350	168
7.6	193	95	222	95
	230	63	238	63
	205	102	281	102
8.0	308	117	346	111
	326	134	373	124
	400	96	395	87
8.4	163	104	217	87
	200	50	249	53
	113	57	300	108
8.8	106	45	187	53
	91	73	254	109
	126	81	207	80
9.2	154	88	249	81
	126	98	273	96
	130	83	333	110
9.6	100	52	184	51
	86	84	212	77
	59	159	223	103
10.0	189	70	249	53
	94	89	193	60
	94	68	236	68
OCaASW				
6.0	259	64	299	100
	274	76	338	125
	301	83	351	133
6.4	347	117	420	183
	371	113	399	196
	353	124	405	180
6.8	321	123	387	168
	342	145	391	202
	319	133	361	177
7.2	276	135	300	155
	260	142	304	170

	280	158	298	169
7.6	210	138	220	117
	233	129	268	130
	219	120	240	122
8.0	250	137	285	123
	264	132	293	114
	220	129	301	128
8.4	209	92	339	106
	222	83	352	103
	177	89	341	120
8.8	217	94	364	110
	225	113	357	114
	230	106	369	106
9.2	218	86	399	99
	210	93	386	104
	205	81	391	100
9.6	244	71	444	86
	249	54	459	72
	257	58	465	75
10.0	145	50	451	90
	166	66	439	95
	171	48	460	81

Table Nineteen - The Effect Of Extracellular pH On The Intracellular pH Of Sperm

Extracellular pH	Fluorescence Readings 495nm	450nm	Fluorescence After Digitonin Readings 495nm	+450
pH5.0				
6.0	259	94	271	103
	252	83	284	97
	257	87	289	100
6.4	251	102	293	110
	287	90	310	88
	263	87	314	96
6.8	297	79	328	75
	267	116	357	119
	293	129	319	115
7.2	239	115	370	121
	237	83	375	100
	241	82	369	95
7.6	253	81	381	86
	287	145	359	113
	231	105	385	107
8.0	295	88	397	80
	272	72	408	76
	274	89	400	85
8.4	302	107	449	93
	226	66	437	81
	261	127	430	107
8.8	238	95	466	97
	269	128	481	108
	256	109	469	102
9.2	220	168	480	121
	217	141	495	119
	223	148	490	118
9.6	123	74	504	108
	115	78	474	106
	119	61	500	101
10.0	138	66	566	105
	157	74	539	99
	184	52	550	80
pH6.0				
6.0	323	68	299	77
	333	81	288	88
	328	70	293	75
6.4	318	84	300	90
	320	84	290	87
	310	80	289	85
6.8	361	97	263	72
	370	74	270	55
	312	93	260	79
7.2	232	64	251	63
	247	63	283	68

	252	83	249	75
7.6	254	79	296	74
	280	75	302	69
	262	80	300	79
8.0	230	80	311	80
	249	79	311	73
	255	81	315	78
8.4	272	81	360	75
	275	76	372	75
	265	88	364	83
8.8	246	96	382	87
	250	102	380	93
	240	101	385	93
9.2	251	64	401	69
	253	76	417	75
	270	61	408	78
9.6	249	69	428	69
	254	73	435	75
	264	83	440	78
10.0	220	90	450	85
	200	93	470	95
	243	108	465	93
pH7.0				
6.0	261	69	276	103
	251	71	275	105
	249	70	280	109
6.4	269	71	289	96
	281	66	290	80
	279	66	300	85
6.8	298	67	247	65
	281	59	263	59
	306	70	269	63
7.2	321	95	238	71
	332	77	236	55
	333	73	239	53
7.6	200	79	247	88
	196	35	252	43
	198	67	255	81
8.0	176	75	293	101
	193	53	310	75
	188	53	312	75
8.4	222	48	345	63
	160	49	361	85
	193	51	370	81
8.8	134	29	399	71
	152	40	403	82
	138	49	400	100
9.2	186	37	419	65
	141	41	410	83

	158	43	433	85
9.6	189	41	467	73
	211	32	450	55
	196	36	455	61
10.0	232	39	475	60
	228	39	500	63
	267	42	503	60

Table Twenty - The Intracellular pH Of Sperm

Extracellular pH	Fluorescence 495nm	Readings 450nm	Fluorescence After Digitonin 495nm	Readings 450nm
6.0	177	48	244	88
	151	44	280	117
	58	14	297	100
6.4	141	41	177	86
	184	30	230	44
	109	26	196	65
6.8	126	31	107	35
	147	56	96	57
	118	31	113	40
7.2	119	25	109	27
	181	61	124	53
	95	27	117	41
7.6	208	58	195	59
	247	73	208	66
	161	34	178	39
8.0	140	48	128	42
	119	69	138	78
	121	39	115	36
8.4	154	34	278	68
	137	22	309	46
	163	72	140	50
8.8	174	91	205	81
	111	31	361	88
	101	46	299	76
9.2	306	161	339	113
	264	127	294	87
	279	204	111	49
9.6	314	90	288	58
	300	163	306	96
	196	265	217	101
10.0	201	107	333	93
	242	49	360	58
	196	60	418	88

Table Twenty-One - The Effect Of Amiloride On The Intracellular pH Of Sperm

Extracellular pH	Fluorescence Readings 495nm	450nm	Fluorescence After Digitonin 495nm	Readings 450nm
6.0	364	94	388	121
	297	84	405	138
	316	81	360	110
6.4	180	83	299	186
	150	45	391	140
	135	49	345	157
6.8	198	41	263	58
	186	36	214	43
	265	70	309	88
7.2	277	68	375	97
	344	89	398	105
	386	92	406	100
7.6	247	67	365	128
	300	85	450	186
	223	89	339	151
8.4	251	142	244	121
	223	97	281	116
	237	67	336	90
8.8	175	41	361	76
	162	54	171	50
	150	51	299	88
9.2	110	36	139	38
	160	28	156	25
	177	117	107	56
9.6	288	193	315	145
	259	84	377	103
	315	222	308	150
10.0	120	62	107	40
	125	102	113	57
	119	70	100	30

Table Twenty-Two - The Effect Of Monensin On The Intracellular pH Of Sperm

Extracellular pH	Fluorescence Readings 495nm	450nm	Fluorescene After Digitonin Readings 495nm	450nm
6.0	130	38	108	53
	120	28	119	38
	133	46	150	76
6.4	113	42	134	82
	106	45	147	94
	124	60	128	112
6.8	138	41	159	63
	125	46	139	68
	140	46	121	54
7.2	177	68	183	94
	135	57	177	103
	195	54	218	74
7.6	144	65	209	123
	132	53	184	97
	108	41	228	109
8.0	113	40	136	59
	102	46	114	66
	129	49	135	63
8.4	272	123	330	172
	261	99	285	121
	282	116	361	168
8.8	194	79	216	90
	218	133	187	118
	193	78	208	86
9.2	237	105	229	97
	225	111	214	100
	249	103	265	105
9.6	280	277	289	220
	250	269	250	201
	279	251	245	188
10.0	200	153	198	103
	214	97	234	86
	238	153	201	96

Table Twenty-Three - The Effect Of Vanadate On Coupled And Uncoupled Respiration

Abeorbance at 340nm	No Of Sperm x 10^8	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^8	No Divisions / min
-FCCP					
ASW			±1μM		
0.471	1.5	5.930	0.440	1.4	2.327
0.502	1.6	5.115	0.565	1.8	2.336
0.502	1.6	5.240	0.267	0.85	1.338
0.565	1.8	6.975	0.471	1.5	2.522
0.691	2.2	8.642	0.345	1.1	1.407
0.314	1.0	3.222	0.408	1.3	1.802
±10μM			±100μM		
0.345	1.1	1.151	0.534	1.7	0.811
0.565	1.8	1.894	0.502	1.6	0.352
0.597	1.9	1.623	0.314	1.0	0.199
0.785	2.5	2.120	0.267	0.85	0.393
0.942	3.0	2.670	0.345	1.1	0.235
0.879	2.8	2.828	0.345	1.1	0.502
+FCCP					
+1μM			±10μM		
0.565	1.8	3.159	0.597	1.9	2.157
0.722	2.3	3.071	0.314	1.0	1.116
0.754	2.4	4.008	0.314	1.0	1.042
0.440	1.4	1.848	0.471	1.5	1.332
0.440	1.4	1.988	0.408	1.3	1.251
0.314	1.0	1.770	0.377	1.2	1.043
±100μM					
0.314	1.0	0.473			
0.408	1.3	0.451			
0.408	1.3	0.403			
0.314	1.0	0.420			
0.440	1.4	0.714			
0.345	1.1	0.440			

Table Twenty-Four - The Effect Of ATP Depletion On Motility

The concentration of sperm was adjusted to 1 x 10^8 sperm/ml in all experiments.

	% Motility		% Motility
Control		+ Antimycin a	
	86		33
	89		33
	84		33
	82		33
	92		33
	78		33
	88		33
	85		32
	85		33
	81		34

Table Twenty-Five - The Effect Of Caffeine (6mM) On The Motility And Oxygen Uptake

Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min
Control				+ Caffeine			
0.345	1.1	82	4.516	0.408	1.3	99	9.786
0.565	1.8	89	7.317	0.377	1.2	98	8.954
0.597	1.9	88	7.192	0.502	1.6	98	11.197
0.330	1.05	81	4.016	0.534	1.7	98	12.769
0.298	0.95	84	3.601	0.314	1.0	99	6.932
0.440	1.4	86	5.740	0.314	1.0	98	6.949

Table Twenty-Six - The Effect Of Caffeine On The Motility Of Sperm

The concentration of sperm was adjusted to 1 x 10^8 sperm/ml in all experiments

Time	% Motility Control	+ Caffeine	Time	% Motility Control	+Caffeine
0h	88	84	0.5h	80	96
	89	87		80	93
	86	89		82	97
	84	82		79	95
	81	84		81	94
	82	86		79	98
1h	68	98	1.5h	59	96
	62	95		56	96
	63	99		60	96
	67	99		55	97
	64	97		57	93
	66	98		61	97
2h	45	91	2.5h	41	88
	43	87		39	85
	43	88		42	87
	46	86		42	83
	44	92		45	90
	46	90		41	85
3h	37	84			
	39	80			
	37	83			
	37	85			
	36	85			
	36	86			

Table Twenty-Seven- The Effect Of Theophylline On The Motility Of Sperm

The concentration of sperm was adjusted to 1 x 10^8 sperm/ml in all experiments.

	% Motility		% Motility		% Motility
Control	83	+10mM	90	+20mM	93
	84		88		93
	82		89		93
	80		87		93
	79		91		94
	85		90		90
	77		89		92
	81		89		94
	81		89		94
	78		88		94
+40mM	96	+50mM	100		
	93		100		
	94		98		
	96		99		
	97		99		
	95		99		
	98		99		
	97		96		
	95		99		
	96		98		

Table Twenty-Eight - The Effect Of Theophylline On The Oxygen Uptake Of Sperm

Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min
Control					
0min			5min		
0.314	1.0	2.630	0.283	0.9	2.970
0.377	1.2	2.844	0.565	1.8	5.220
0.345	1.1	2.970	0.754	2.4	7.752
0.345	1.1	2.530	0.722	2.3	6.831
0.408	1.3	3.705	0.345	1.1	3.740
0.440	1.4	3.024	0.440	1.4	4.004
0.314	1.0	2.480	0.345	1.1	3.410
0.314	1.0	2.520	0.377	1.2	3.768
0.377	1.2	3.192	0.314	1.0	3.350
0.471	1.5	3.495	0.314	1.0	2.950
10min			15min		
0.345	1.1	4.180	0.314	1.0	3.800
0.377	1.2	3.840	0.314	1.0	3.800
0.377	1.2	4.320	0.314	1.0	4.100
0.377	1.2	4.080	0.314	1.0	3.500
0.377	1.2	4.920	0.314	1.0	3.700
0.377	1.2	3.480	0.314	1.0	3.900
0.345	1.1	4.070	0.314	1.0	4.000
0.345	1.1	3.520	0.314	1.0	3.600
0.440	1.4	5.040	0.314	1.0	3.800
0.471	1.5	5.250	0.314	1.0	4.000
20min			25min		
0.345	1.1	4.675	0.440	1.4	5.838
0.440	1.4	5.320	0.471	1.5	6.660
0.565	1.8	7.380	0.471	1.5	7.065
0.314	1.0	3.900	0.471	1.5	5.820
0.283	0.9	3.870	0.534	1.7	7.395
0.251	0.8	2.920	0.565	1.8	8.082
0.314	1.0	4.400	0.345	1.1	5.093
0.345	1.1	3.993	0.345	1.1	4.367
0.345	1.1	4.378	0.408	1.3	5.720
0.502	1.6	6.400	0.534	1.7	7.140
+20mM					
0min			5min		
0.314	1.0	3.190	0.408	1.3	6.045
0.345	1.1	3.091	0.502	1.6	6.320
0.408	1.3	3.978	0.659	2.1	10.143
0.408	1.3	3.952	0.314	1.0	3.770
0.440	1.4	4.480	0.314	1.0	4.400
0.314	1.0	2.800	0.345	1.1	4.620
0.283	0.9	2.862	0.408	1.3	5.876
0.345	1.1	3.102	0.314	1.0	4.070
0.408	1.3	3.887	0.314	1.0	3.900
0.440	1.4	4.242	0.314	1.0	4.700
10min			15min		
0.502	1.6	8.800	0.408	1.3	6.812
0.534	1.7	7.599	0.345	1.1	6.787
0.597	1.9	8.797	0.377	1.2	6.876
0.659	2.1	11.340	0.345	1.1	6.237
0.597	1.9	10.070	0.345	1.1	6.479
0.534	1.7	8.007	0.314	1.0	5.530
0.565	1.8	7.740	0.314	1.0	5.610
0.345	1.1	6.270	0.314	1.0	5.770
0.377	1.2	6.228	0.314	1.0	6.010
0.565	1.8	8.640	0.408	1.3	6.994
20min			25min		
0.471	1.5	0.620	0.345	1.1	5.093

0.502	1.6	8.512	0.377	1.2	5.352
0.565	1.8	9.630	0.408	1.3	5.603
0.628	2.0	10.100	0.440	1.4	6.566
0.691	2.2	12.012	0.471	1.5	6.225
0.345	1.1	5.434	0.534	1.7	8.245
0.408	1.3	6.643	0.565	1.8	8.118
0.502	1.6	8.448	0.345	1.1	4.939
0.534	1.7	8.840	0.314	1.0	4.750
0.565	1.8	9.540	0.345	1.1	4.774
+40mM					
0min			5min		
0.314	1.0	2.850	0.377	1.2	8.124
0.345	1.1	2.811	0.440	1.4	9.002
0.440	1.4	4.228	0.408	1.3	8.463
0.408	1.3	3.224	0.502	1.6	10.704
0.565	1.8	4.554	0.565	1.8	12.438
0.597	1.9	5.586	0.345	1.1	7.084
0.314	1.0	2.700	0.314	1.0	6.830
0.283	0.9	2.502	0.408	1.3	8.060
0.345	1.1	2.981	0.314	1.0	6.660
0.377	1.2	2.952	0.345	1.1	6.853
10min			15min		
0.314	1.0	8.360	0.502	1.6	14.896
0.314	1.0	9.040	0.440	1.4	12.712
0.314	1.0	9.300	0.345	1.1	9.900
0.314	1.0	8.190	0.251	0.8	7.456
0.314	1.0	8.820	0.251	0.8	7.400
0.314	1.0	8.500	0.251	0.8	7.392
0.314	1.0	9.000	0.408	1.3	12.025
0.314	1.0	8.400	0.345	1.1	10.065
0.314	1.0	8.280	0.314	1.0	9.080
0.314	1.0	9.120	0.345	1.1	10.120
20min			40min		
0.314	1.0	8.320	0.377	1.2	9.060
0.314	1.0	7.680	0.502	1.6	12.192
0.345	1.1	7.799	0.659	2.1	15.540
0.502	1.6	14.096	0.628	2.0	15.200
0.534	1.7	13.175	0.628	2.0	14.600
0.565	1.8	15.030	0.628	2.0	15.000
0.314	1.0	8.500	0.628	2.0	15.080
0.314	1.0	7.520	0.314	1.0	7.380
0.345	1.1	9.548	0.345	1.1	8.140
0.345	1.1	7.920	0.408	1.3	9.880
+50mM					
0min			5min		
0.314	1.0	3.450	0.314	1.0	7.500
0.314	1.0	3.200	0.314	1.0	7.480
0.314	1.0	3.260	0.314	1.0	7.600
0.314	1.0	3.300	0.314	1.0	7.140
0.314	1.0	3.470	0.314	1.0	7.250
0.314	1.0	3.500	0.314	1.0	7.500
0.314	1.0	3.130	0.314	1.0	7.500
0.314	1.0	3.290	0.314	1.0	7.750
0.314	1.0	3.310	0.314	1.0	7.360
0.314	1.0		0.314	1.0	7.600
10min			15min		
0.345	1.1	11.066	0.471	1.5	18.900
0.345	1.1	10.923	0.502	1.6	19.920
0.408	1.3	12.831	0.534	1.7	20.502
0.377	1.2	11.760	0.659	2.1	26.124
0.502	1.6	15.52	0.628	2.0	24.720
0.534	1.7	16.320	0.502	1.6	19.664
0.565	1.8	18.018	0.534	1.7	22.100
0.345	1.1	10.890	0.628	2.1	23.320
0.408	1.3	12.740	0.345	1.1	13.420

0.345	1.1	11.00	0.314	1.0	12.430
20min			25min		
0.314	1.0	12.050	0.314	1.0	10.280
0.314	1.0	11.850	0.345	1.1	11.033
0.314	1.0	11.770	0.345	1.1	10.989
0.314	1.0	12.130	0.565	1.8	18.558
0.314	1.0	11.970	0.597	1.9	19.228
0.314	1.0	11.930	0.691	2.2	22.308
0.314	1.0	12.085	0.345	1.1	11.099
0.314	1.0	12.018	0.408	1.3	13.221
0.314	1.0	11.815	0.440	1.4.	14.280
0.314	1.0	11.885	0.440	1.4	14.084

Table Twenty-Nine - The Effect Of pH On The Motility Of Sperm

The concentration of sperm was adjusted to 1 x 10^7 sperm/ml in all experiments.

pH	% MOTILITY	pH	% MOTILITY	pH	% MOTILITY	pH	% MOTILITY	pH	% MOTILITY
6.0	32	6.4	42	6.8	40	7.2	55	9.2	34
	25		30		44		49		32
	30		32		42		54		30
	30		34		42		54		28
	28		38		46		50		33
	31		44		38		52		29
	29		36		43		53		36
	29		32		41		51		31
	32		40		42		50		32
	31		42		43		52		26
7.6	68	8.0	92	8.4	88	8.8	60		
	71		87		76		53		
	70		82		83		50		
	68		88		77		54		
	69		86		76		59		
	70		83		80		56		
	68		85		83		51		
	68		91		84		47		
	69		88		77		63		
	69		86		80		57		

Table Thirty - The Effect Of dbcAMP (5mM) On The Motility Of Sperm

The concentration of sperm was adjusted to 1 x 10^8 sperm/ml in all experiments.

	% Motility		% Motility
Control	87	+dbcAMP	100
	82		100
	83		99
	86		99
	86		99
	84		99

Table Thirty-One - The Effect Of dbcAMP On pH - Dependent Motility

pH	% Motility -dbcAMP	+dbcAMP	pH	% Motility -dbcAMP	+dbcAMP
6.0	32	67	6.5	26	77
	30	60		42	64
	28	66		29	61
	33	69		27	74
	29	59		32	77
	31	65		39	60
	27	61		36	62
	30	64		41	76
	32	62		26	78
	28	57		42	61
7.0	44	700	7.5	75	84
	43	81		62	89
	45	67		61	83
	34	78		59	92
	37	69		74	86
	33	83		71	82
	40	80		65	93
	35	72		77	87
	38	73		72	94
	41	77		64	90
8.0	89	99			
	85	100			
	91	98			
	88	98			
	87	95			
	83	97			
	86	99			
	84	99			
	90	96			
	87	97			

Table Thirty-Two - The Effect Of dbcAMP On The Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^6	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^6	No Divisions / min
Control			±0.1mM		
0.593	1.89	7.995	0.408	1.3	12.026
0.597	1.9	6.954	0.471	1.5	11.834
0.455	1.45	5.989	0.471	1.5	12.977
0.345	1.1	4.136	0.440	1.4	11.885
0.408	1.3	5.018	0.377	1.2	10.861
0.565	1.8	7.254	0.283	0.9	7.280
0.597	1.9	8.227	0.754	2.4	21.002
0.659	2.1	7.476	0.581	1.85	15.150
0.345	1.1	4.351	0.612	1.95	16.712
0.314	1.0	3.935	0.440	1.4	11.998
±1mM			±5mM		
0.502	1.6	17.677	0.565	1.8	25.992
0.408	1.3	15.922	0.565	1.8	24.372
0.502	1.6	18.766	0.659	2.1	29.971
0.471	1.5	17.351	0.345	1.1	15.079
0.440	1.4	16.293	0.471	1.5	21.408
0.471	1.5	17.487	0.440	1.4	19.191
0.471	1.5	17.772	0.534	1.7	23.792
0.345	1.1	12.293	0.502	1.6	22.376
0.314	1.0	11.648	0.377	1.2	17.126
0.408	1.3	15.142	0.440	1.4	19.191
±10mM					
0.377	1.2	13.536			
0.345	1.1	11.022			
0.440	1.4	14.903			
0.440	1.4	14.896			
0.471	1.5	15.269			
0.345	1.1	12.233			
0.377	1.2	12.786			
0.314	1.0	10.660			
0.345	1.1	11.197			
0.345	1.1	12.233			

Table Thirty-Three - The Effect Of Forskolin On The Oxygen Uptake Of Sperm

Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^8	No Divisions / min
Control			±1mM		
0..754	2.4	9.571	0.471	1.5	9.308
0.879	2.8	10.366	0.502	1.6	9.960
0.565	1.8	7.004	0.534	1.7	10.277
0.597	1.9	7.522	0.534	1.7	10.566
0.659	2.1	7.978	0.440	1.4	8.435
0.691	2.2	8.208	0.345	1.1	6.639
0.722	2.3	8.671	0.314	1.0	6.030
0.440	1.4	5.488	0.283	0.9	5.427
0.565	1.8	7.261	0.565	1.8	11.196
0.314	1.0	3.656	0.440	1.4	8.708
±10mM			±50mM		
0.314	1.0	7.018	0.314	1.0	11.482
0..408	1.3	8.473	0.377	1.2	12.292
0.440	1.4	9.668	0.377	1.2	11.064
0.534	1.7	11.271	0.314	1.0	9.415
0.471	1.5	10.320	0.283	0.9	8.515
0.502	1.6	10.650	0.408	1.3	13.256
0.534	1.7	12.301	0.440	1.4	14.461
0.408	1.3	8.190	0.471	1.5	15.345
0.440	1.4	9.906	0.502	1.6	15.086
0.440	1.4	9.044	0.534	1.7	15.859
±100mM			±500mM		
0.314	1.0	15.079	0.408	1.3	13.252
0.377	1.2	16.861	0.502	1.6	14.774
0.345	1.1	16.339	0.534	1.7	16.718
0.534	1.7	24.274	0.816	2.6	24.944
0.691	2.2	32.846	0.628	2.0	20.494
0.754	2.4	35.446	0.879	2.8	25.707
0.345	1.1	15.620	0.754	2.4	23.474
0.628	2.0	28.722	0.659	2.1	20.259
0.314	1.0	14.416	0.628	2.0	20.028
0.471	1.5	22.071	0.534	1.8	16.945
±1mM					
0.314	1.0	9.330			
0.314	1.0	8.818			
0.345	1.1	10.019			
0.440	1.4	12.656			
0.502	1.6	14.750			
0.534	1.7	15.179			
0.597	1.9	17.621			
0.659	2.1	18.635			
0.691	2.2	20.161			
0.722	2.3	20.663			

Table Thirty-Four - The Effect Of PMA (1nM) On The Motility Of Sperm

Absorbance at 340nm	No Of Sperm x 10^8	% Motility	Absorbance at 340nm	No Of Sperm x 10^8	% Motility
Control			±PMA		
0.440	1.4	83	0.377	1.2	100
0.565	1.8	84	0.377	1.2	98
0.659	2.1	80	0.377	1.2	97
0.314	1.0	79	0.691	2.2	98
0.314	1.0	82	0.942	3.0	98
0.471	1.5	81	0.879	2.8	98
0.565	1.8	78	0.659	2.1	99
0.534	1.7	83	0.942	3.0	96
0.283	0.9	81	0.848	2.7	98
0.471	1.5	78	0.848	2.7	97

Table Thirty-Five - The Effect Of PMA (1nM) On The Motility And Oxygen Uptake Of Sperm Incubated In OCaASW

Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min
OCaASW				+PMA			
0.345	1.1	28	1.488	0.314	1.0	31	1.480
0.408	1.3	29	1.198	0.314	1.0	31	1.920
0.267	0.85	30	1.140	0.314	1.0	27	2.033
0.283	0.9	20	1.285	0.314	1.0	28	1.834
0.283	0.9	26	1.401	0.314	1.0	26	1.750
0.314	1.0	24	1.546	0.314	1.0	29	1.649
0.314	1.0	27	1.505	0.314	1.0	29	1.760
0.502	1.6	23	1.390	0.314	1.0	30	1.360
0.314	1.0	21	1.181	0.314	1.0	33	1.640
0.314	1.0	22	1.296	0.314	1.0	25	1.570

Table Thirty-Six - The Effect Of PMA On The Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^8	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^8	No Divisions / min
Control			±0.1nM		
0.314	1.0	4.230	0.597	1.9	16.122
0.534	1.7	6.222	0.534	1.7	12.691
0.565	1.8	7.434	0.345	1.1	9.158
0.597	1.9	7.144	0.267	0.85	6.481
0.659	2.1	8.106	0.283	0.9	7.210
0.471	1.5	6.045	0.314	1.0	7.939
0.440	1.4	6.062	0.345	1.1	8.817
0.408	1.3	4.628	0.471	1.5	11.903
0.377	1.2	4.746	0.440	1.4	11.417
0.377	1.2	4.722	0.314	1.0	7.795
±0.5nM			±1nM		
0.314	1.0	9.430	0.879	2.8	30.640
0.345	1.1	9.537	0.345	1.1	11.377
0.345	1.1	10.093	0.471	1.5	15.971
0.314	1.0	8.925	0.785	2.5	26.598
0.314	1.0	9.060	0.345	1.1	12.042
0.283	0.9	8.136	0.314	1.0	10.339
0.440	1.4	12.810	0.659	2.1	22.989
0.612	1.95	17.453	0.628	2.0	21.296
0.722	2.3	22.630	0.565	1.8	19.148
0.534	1.7	14.044	0.597	1.9	19.644
±10nM			±50nM		
0.471	1.5	12.761	0.471	1.5	10.356
0.502	1.6	12.971	0.345	1.1	7.102
0.471	1.5	12.468	0.440	1.4	9.386
0.597	1.9	15.774	0.942	3.0	19.668
0.722	2.3	18.850	0.659	2.1	14.288
0.597	1.9	15.783	0.565	1.8	11.621
0.267	0.85	7.069	0.534	1.7	11.424
0.345	1.1	9.127	0.565	1.8	11.772
0.408	1.3	10.799	0.502	1.6	10.474
0.408	1.3	11.114	0.345	1.1	7.385
±100nM					
0.314	1.0	5.874			
0.314	1.0	5.526			
0.267	0.85	4.929			
0.283	0.9	5.198			
0.345	1.1	6.161			
0.314	1.0	5.808			
0.785	2.5	14.063			
0.314	1.0	5.592			
0.345	1.1	6.343			
0.534	1.7	9.578			

Table Thirty-Seven - The Effect Of BPDD On The Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^8	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^8	No Divisions / min
Control			+0.1nM		
0.314	1.0	3.941	0.330	1.05	8.710
0.314	1.0	3.597	0.314	1.0	8.400
0.565	1.8	6.543	0.565	1.8	13.311
0.345	1.1	3.960	0.659	2.1	16.643
0.408	1.3	5.135	0.314	1.0	7.290
0.345	1.1	3.891	0.440	1.4	10.871
±1nM			±10nM		
0.502	1.6	22.774	0.408	1.3	13.202
0.534	1.7	22.770	0.408	1.3	12.721
0.565	1.8	24.343	0.314	1.0	9.827
0.565	1.8	25.513	0.267	0.85	8.630
0.722	2.3	32.439	0.283	0.9	9.132
0.659	2.1	28.253	0.565	1.8	17.555

Table Thirty-Eight - The Effect Of H-7 On The Motility And Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min
Control				0.5ug/ml			
0.314	1.0	86	2.655	1.3	4.14	71	9.800
0.314	1.0	87	3.051	1.3	4.14	75	11.150
0.314	1.0	81	3.240	1.3	4.14	68	10.595
0.314	1.0	86	3.149	1.3	4.14	81	11.250
0.314	1.0	83	3.980	1.3	4.14	69	9.710
0.314	1.0	82	3.215	1.3	4.14	77	10.499
10ug/ml				15ug/ml			
0.610	1.94	58	5.135	0.314	1.0	44	0.957
0.610	1.94	55	4.141	0.314	1.0	30	1.697
0.610	1.94	68	4.000	0.314	1.0	35	0.9797
0.610	1.94	57	3.789	0.314	1.0	33	0.756
0.610	1.94	57	4.300	0.314	1.0	44	0.920
0.610	1.94	63	4.322	0.314	1.0	34	1.070
20ug/ml				30ug/ml			
0.314	1.0	20	0.448	0.314	1.0	19	0.245
0.314	1.0	17	0.326	0.314	1.0	21	0.311
0.314	1.0	30	0.470	0.314	1.0	18	0.463
0.314	1.0	25	0.389	0.314	1.0	17	0.366
0.314.	1.0	30	0.330	0.314	1.0	20	0.547
0.314	1.0	20	0.389	0.314	1.0	18	0.386

Table Thirty-Nine - The Effect Of H-7 On PMA Stimulated Sperm

Absorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min
Control				+PMA			
0.597	1.9	86	6.726	0.330	1.05	100.	11.526
0.565	1.8	87	7.182	0.377	1.2	100	12.383
0.345	1.1	81	4.274	0.440	1.4	100	15.190
0.377	1.2	83	4.380	0.597	1.9	99	19.847
0.408	1.3	83	4.411	0.345	1.1	97	11.282
0.471	1.5	85	6.206	0.377	1.2	98	13.096
0.502	1.6	81	5.520	0.283	0.9	99	9.300
0.345	1.1	82	4.271	0.408	1.3	99	14.365
0.408	1.3	87	4.741	0.408	1.3	98	14.044
0.377	1.2	87	4.896	0.345	1.1	99	11.531
+PMA + H7							
0.345	1.1	30	0.495				
0.377	1.2	27	0.600				
0.408	1.3	28	1.040				
0.345	1.1	27	1.012				
0.345	1.1	30	0.935				
0.314	1.0	29	0.380				
0.283	0.9	29	0.594				
0.754	2.4	26	1.056.				
0.345	1.1	28	0.946				
0.314	1.0	26	0.620				

Table Forty - The Effect Of EGTA On The Motility And Oxygen Uptake Of Sperm

Absorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min	Absorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min
Control				+0.01mM			
0.314	1.0	96	3.855	0.597	1.9	77	5.966
0.314	1.0	97	3.552	0.314	1.0	73	2.486
0.314	1.0	96	3.461	0.597	1.9	74	4.722
0.314	1.0	91	3.764	0.597	1.9	72	5.926
0.314	1.0	91	3.864	0.754	2.4	76	7.538
0.314	1.0	92	3.500	0.942	3.0	73	7.521
0.314	1.0	95	3.902	0.267	0.85	75	2.587
0.314	1.0	93	3.412	0.314	1.0	78	2.583
0.314	1.0	91	3.826	0.440	1.4	78	3.513
0.314	1.0	96	3.460	0.471	1.5	75	4.676
+0.1mM				+1mM			
0.471	1.5	39	4.428	0.534	1.7	32	3.871
0.345	1.1	35	2.746	0.659	2.1	24	3.866
0.283	0.9	31	2.228	0.345	1.1	22	1.948
0.754	2.4	43	7.058	0.267	0.85	30	1.865
0.502	1.6	41	4.757	0.754	2.4	26	4.618
0.377	1.2	44	3.018	0.973	3.1	34	7.270
0.565	1.8	33	4.459	0.848	2.8	31	6.334
0.440	1.4	30	4.106	0.848	2.7	25	5.969
0.502	1.6	36	4.754	0.471	1.5	25	2.784
0.471	1.5	34	3.761	0.597	1.9	31	3.610
+10mM							
0.314	1.0	18	1.277				
0.314	1.0	15	1.326				
0.314	1.0	17	0.880				
0.314	1.0	17	1.171				
0.314	1.0	19	0.929				
0.314	1.0	18	1.258				
0.314	1.0	17	1.080				
0.314	1.0	15	1.251				
0.314	1.0	16	0.903				
0.314	1.0	15	0.955				

Table Forty-One - The Effect Of EGTA On The Motility And Oxygen Uptake Of Sperm Incubated In OCaASW

Abeorbance at 340nm	No Of Sperm x 10^6	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^6	% Motility	No Divisions / min
±EGTA				±Ca			
0.502	1.6	2	0.375	0.314	1.0	17	0.915
0.565	1.8	0	0.150	0.251	0.8	18	0.950
0.314	1.0	2	0.330	0.534	1.7	14	0.760
0.377	1.2	2	0.400.	0.534	1.7	16	0.560
0.408	1.3	3	0.190	0.628	2.0	16	0.590
0.534	1.7	2	0.123	0.502	1.6	15	0.750

Table Forty-Two - The Effect Of A23187 On The Motility And Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^6	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^6	% Motility	No Divisions / min
ASW				±A23187			
0.314	1.0	86	3.500	0.60	1.91	99	13.122
0.314	1.0	84	3.704	0.60	1.91	99	13.567
0.314	1.0	84	3.401	0.60	1.91	100	12.710
0.314	1.0	82	3.578	0.60	1.91	99	13.080
0.314	1.0	85	3.794	0.60	1.91	98	13.300
0.314	1.0	83	3.239	0.60	1.91	98	12.995
OCaASW				±A23187			
0.660	2.1	27	2.730	0.89	2.84	29	3.709
0.660	2.1	23	3.166	0.89	2.84	27	4.711
0.660	2.1	25	2.335	0.89	2.84	32	4.499
0.660	2.1	20	2.890	0.89	2.84	24	4.600
0.660	2.1	24	2.660	0.89	2.84	26	4.085
0.660	2.1	21	2.700	0.89	2.84	30	4.850
ONaASW				±A23187			
0.55	1.75	1	1.208	0.56	1.79	80	3.780
0.55	1.75	1	1.154	0.56	1.79	86	4.456
0.55	1.75	2	1.360	0.56	1.79	81	3.510
0.55	1.75	0	1.110	0.56	1.79	90	4.080
0.55	1.75	0	1.200	0.56	1.79	95	3.375
0.55	1.75	2	1.225	0.56	1.79	93	3.658

Table Forty-Three - The Effect Of Fura -2- AM On The Motility And Oxygen Uptake Of Sperm

Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min	Abeorbance at 340nm	No Of Sperm x 10^8	% Motility	No Divisions / min
Control							
0h				0.5h			
0.597	1.9	94	6.774	0.502	1.6	88	5.184
0.659	2.1	90	7.959	0.597	1.9	87	6.213
0.345	1.1	80	4.109	0.659	2.1	84	6.846
0.267	0.85	98	3.149	0.691	2.2	89	7.128
0.597	1.9	76	6.669	0.597	1.9	83	6.137
0.754	2.4	72	8.628	0.565	1.8	85	6.012
1h				1.5h			
0.157	0.5	83	1.575	0.314	1.0	71	3.256
0.314	1.0	84	2.855	0.408	1.3	85	4.134
0.502	1.6	84	4.664	0.408	1.3	81	3.987
0.471	1.5	83	4.763	0.597	1.9	88	5.761
0.471	1.5	83	4.613	0.659	2.1	68	6.531
0.283	0.9	82	2.660	0.314	1.0	75	3.223
2h				2.5h			
0.597	1.9	70	5.700	0.377	1.2	54	3.863
0.502	1.6	69	4.328	0.314	1.0	61	3.051
0.345	1.1	65	2.860	0.345	1.1	57	2.762
0.534	1.7	64	4.956	0.565	1.8	52	5.535
0.408	1.3	71	3.764	0.314	1.0	66	2.679
0.408	1.3	66	3.504	0.314	1.0	64	2.595
3h				3.5h			
0.314	1.0	50	2.755	0.471	1.5	45	3.750
0.345	1.1	50	2.932	0.534	1.7	45	3.949
0.157	0.5	49	1.355	0.659	2.1	45	5.240
0.267	0.85	51	2.155	0.157	0.5	47	1.189
0.283	0.9	52	2.241	0.314	1.0	43	2.425
0.565	1.8	48	4.401	0.345	1.1	46	2.640
4h							
0.314	1.0	33	2.400				
0.314	1.0	34	2.390				
0.314	1.0	31	2.250				
0.314	1.0	32	2.000				
0.314	1.0	33	2.158				
0.314	1.0	33	2.010				
+FURA - 2 -AM							
0h				0.5h			
0.377	1.2	90	5.082	0.314	1.0	87	3.795
0.408	1.3	88	5.524	0.659	2.1	89	6.899
0.440	1.4	92	4.988	0.502	1.6	85	5.872
0.440	1.4	80	5.810	0.314	1.0	79	3.310
0.345	1.1	82	3.904	0.314	1.0	81	3.800
0.345	1.1	84	3.960	0.283	0.9	83	3.105
1h				1.5h			
0.314	1.0	83	3.483	0.377	1.2	80	3.798
0.314	1.0	81	3.472	0.502	1.6	68	4.928
0.314	1.0	85	3.310	0.565	1.8	83	5.940
0.314	1.0	75	3.217	0.659	2.1	65	6.311
0.314	1.0	77	3.500	0.345	1.1	77	2.400
0.314	1.0	79	3.280	0.408	1.3	71	3.861
2h				2.5h			
0.314	1.0	62	3.305	0.440	1.4	51	4.655
0.314	1.0	64	3.240	0.534	1.7	58	5.287
0.314	1.0	62	3.120	0.565	1.8	48	6.246
0.314	1.0	62	2.925	0.534	1.7	54	5.432
0.314	1.0	62	2.990	0.534	1.7	61	5.797

0.314	1.0	62	3.110	0.314	1.0	64	3.050
3h				3.5h			
0.471	1.5	45	4.800	0.345	1.1	42	3.311
0.502	1.6	44	5.024	0.597	1.9	41	4.370
0.754	2.4	44	7.200	0.534	1.7	41	3.723
0.408	1.3	44	3.777	0.424	1.35	43	3.915
0.345	1.1	46	3.405	0.314	1.0	40	2.700
0.345	1.1	44	3.245	0.502	1.6	41	4.000
4h							
0.345	1.1	37	2.651				
0.345	1.1	33	2.635				
0.565	1.8	35	4.059				
0.408	1.3	36	3.198				
0.502	1.6	32	3.736				
0.440	1.4	34	3.178				

DX

189643