Ca^{2+}—ATPASE IN BULL SPERMATOZOA

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science
(ANIMAL BIOCHEMISTRY)

TO THE KURUKSHETRA UNIVERSITY
KURUKSHETRA

1982

By

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DIVISION OF DAIRY CHEMISTRY
NATIONAL DAIRY RESEARCH INSTITUTE
(I. C. A. R.)
KARNAL (Haryana) INDIA

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Dated the 12th February, 1982

I certify that the work reported in this dissertation entitled 'Ca^{2+}-ATPase in bull spermatozoa' was carried out by Miss Alka Kamra under my supervision and guidance in partial fulfilment of her M.Sc. (Animal Biochemistry) course.

S Rawand
12/2/84

(S.R.ANAND)
Professor of Biochemistry

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Alka Kamra

(ALKA KAMRA)

C O N T E N T S

CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

INTRODUCTION

Although it is known since long that divalent Ca^{2+} ion is ubiquitous in its occurrence in all variety of cell type, its role in a number of biological processes is beginning to be unravelled at the molecular level only recently. Besides its established role in the contraction of muscle, it is now known to aid in cell motility, axonemal flow, cytoplasmic streaming, chromosome movement, neurotransmitter release, endocytosis and exocytosis.

Excitation of a cell leads to transient increase in the intracellular concentration of calcium which in turn is responsible for eliciting the physiological response. The latter is accomplished by interaction of calcium ions with specific intracellular calcium binding proteins of which calmodulin is perhaps the most ubiquitous. The return of calcium concentration to its basal level is accomplished by a variety of mechanisms which are beginning to be understood. In fact, there appears to be no common mechanism for the transport of Ca across the cell membrane of all tissues and species. Indeed the only cell species about which the mechanism of transport is known in any depth is the red blood cell for which there exists a specific Ca^{2+}-ATPase.

Different pools of Ca exist in both the extracellular and intracellular environments. The concentration of intracellular Ca (10^{-5} to 10^{-8}M) is less by several orders of magnitude, than that existing outside the cells (10^{-3}M). Precisely, how the Ca^{2+} is translocated to the cell interior is largely unknown but in the red blood cell the efflux is recognised to be an active process or "Ca pump". The components of calcium pump has been demonstrated to Ca^{2+}-activated ATPase distinct from Na^{+}, K^{+}-ATPase found in the plasma membrane of animal cells. Calcium ATPase has a low Km with high turnover rate for calcium. Although the energy dependent efflux of calcium ions is an important process contributing to cellular calcium homeostasis, this may not be the sole means by which homeostasis is achieved. Mitochondria and microsomes are two other cell organelles which accumulate calcium ions and require energy. However, the physiological role of Ca^{2+} accumulation by mitochondria has not yet been fully understood.

In recent years, Ca^{2+} has been shown to have an important role in sperm motility and in the process of fertilization. A direct relationship between calcium uptake and motility activation has been described. In presence of agents which alter membrane permeability, two influxes of calcium ions into spermatozoa were

recorded. The influx which sequester Ca^{2+} ions in mitochondria did not affect either the respiratory or kinetic activity but the accumulation of Ca^{2+} in the extramitochondrial region resulted in the activation of sperm motility. Likewise, it was observed that guinea pig spermatozoa incubated in a minimal incubation medium has two influxes of Ca^{2+} ions. The initial uptake was apparently unrelated to capacitation but is associated with spermatozoal surface as revealed by ^{45}Ca uptake experiments. The secondary uptake of Ca^{2+} was observed during incubation under conditions that produce capacitation in vitro and the time course of this paralleled that of acrosome reaction.

The mechanism of calcium influxes into the sperm cell is not yet known. In the present study, we have detected the presence of Ca^{2+}-ATPase and then have studied its intracellular distribution. Some properties of Ca^{2+}-ATPases present in sperm plasma membrane as well as in demembranated sperm cell are also described.

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CHAPTER II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

(1) Occurrence and Localization

Two types of Ca^{2+}-dependent ATPase activities have thus far been described. A Ca^{2+}-Mg^{2+}-ATPase activity has been reported in intestinal smooth muscle (Godfrained, Sturbois & Verbeke, 1976) as well as in myometrium (Akerman and Wikström, 1979). Because of a very high 'basal' activity of ATPase dependent on Mg^{2+} present in different cells, the detection and characterisation of Ca^{2+}-Mg^{2+}-ATPase has been difficult (Carsten, 1969; Janis, Crankshaw and Daniel, 1977; Verity and Bevan, 1969). The second type of ATPase activity found, depends upon Ca^{2+} in the absence of Mg^{2+}. In this category again two types of activities have been reported and characterised by either having a low affinity or high affinity for Ca^{2+}. Ca^{2+}-ATPase having low affinity for Ca^{2+} has been found in plasma membrane of skeletal muscle (McNamara, Sulakhe and Dhalla, 1971), uterus smooth muscle (Shami and Radde, 1971) and microsomes of vascular smooth muscle (Verity and Bevan, 1969). Thorens (1979) first described a Ca^{2+}-ATPase activity with high affinity for Ca^{2+} in smooth muscle. Since then this kind of Ca^{2+}-ATPase has been recognised in tissue of intestinal mucosa (Martin, Melancon and Deluca, 1969), renal

tubules (Parkinson and Hadde, 1971), and rat liver mitochondria (Moore, 1971). In other tissues where Ca^{2+} has a specific function, also possess Ca^{2+}-ATPase. These are sarcoplasmic reticulum (MacLennan, 1970), brain and nerve tissue (Berl and Puszkin, 1970; Nakamaru, Kosakai and Konishi, 1967).

Ca^{2+}-ATPase has been characterised in the red blood cell (Schatzmann and Vincenzi, 1969; Cha, Ohin and Lee, 1971), salivary gland (Watson, Izutsu and Siegel, 1974), gill plasma membrane (Ma et al, 1974), blood platelet membrane (Rooblee, Shepro and Belamarich, 1973), rat kidney cortex (Parkinson and Hadde, 1971) and microsomal fraction of smooth muscle (Wuytack and Casteels, 1980).

(ii)Transport function

The function of this enzyme in the transport of Ca^{2+} is studied relatively in few systems and the two tissues widely investigated are sarcoplasmic reticulum and the erythrocytes. Ebashi and Lipmann (1962) had shown that Ca transport in sacroplasmic reticulum vesicles is tightly coupled to ATP hydrolysis which is catalysed by a membrane bound Ca^{2+}-Mg^{2+}-ATPase. Since then considerable insight has been gained and the information has been reviewed by Hasselbach (1978).

The occurrence of Ca^{2+}-stimulated Mg^{2+}-ATPase as well as the dependence of Ca transport on ATP have

been described in membrane fractions from various smooth muscles (Carsten, 1969; Fitzpatrick et al., 1972; Janis, Crankshaw and Daniel, 1977), in erythrocyte ghosts (Schatzmann, 1967), in mitochondria and microscomes of muscle cells (Martonosi, 1969; Ohnishi and Ebashi, 1964). Several groups of workers have proposed transport of Ca linked to Ca^{2+} stimulated ATPase based on the evidence of cytoplasmic Ca pool and strong concentration gradient at the plasma membrane (Langer, 1968; Rasmussen, 1970; Sonnenblick and Stam, 1969). In mitochondrial system, Ca transport can occur at the expense of energy generated through electron transfer (Brierley, Murer and Green, 1963) and is thus separate from the system involving hydrolysis of ATP. But in sarcoplasmic reticulum, a separation of Ca transport system similar to the one in mitochondria has been achieved and it is conceivable that the ATPase enzyme and the ion transport enzyme are one and the same (Skou, 1965).

A link between membrane bound Ca^{2+}-Mg^{2+}-ATPase and Ca pump localized in plasma membrane has been indicated in human red blood cells (Schatzmann and Vincenzi, 1969; Olson and Cazort, 1969; Lee and Shin, 1969) as well as in other systems (McNamara, Sulakhe and Dhalla, 1971). The enzyme is involved in the active efflux of Ca across the cell membrane by providing the required energy for the 'Ca pump' through the hydrolysis

of ATP. In renal tubules, a common transport mechanism for two divalent ions, Ca^{2+} and Mg^{2+} must operate. The two divalent ions compete with one another for the activation of ATPase in kidney (Parkinson and Radde, 1971).

(III) Reaction mechanism

The mechanism of Ca transport has been explained through the involvement of carrier which is phosphorylated by ATP at the outer surface of microsomal membrane resulting in the creation of a high affinity binding site for Ca. The Ca bound phosphorylated carrier then undergoes a conformational change moving across the membrane (Weber and Sanadi, 1966). At the inner surface, the carrier is dephosphorylated which now has low affinity for Ca and thus is released from the carrier. Repetition of this cycle results in continuous Ca influx and stoichiometrically related ATPase activity. A comparison of rate of ATP hydrolysis with that of Ca accumulation suggested the transport of 2 moles of Ca for each mole of ATP hydrolysed under optimum conditions (Hasselbach and Makinose, 1961, 1962, 1963; Weber, Herz and Reiss, 1966; Yamada, Yamamoto and Tonomura, 1970). Although smaller or larger coupling ratios have been reported but the variations may be as a consequence of using different substrates, inhibitors or different temperatures (Suko and Hasselbach, 1976; Martonosi and Feretos, 1964; Hasselbach and Makinose, 1961).

In sarcoplasmic reticulum, the enzyme is phosphorylated by the transfer of terminal phosphate of ATP to an aspartate residue of the enzyme protein yielding an acid stable phosphate bond (Yamamoto and Tonomura, 1968; Makinose, 1969; Inesi et al., 1970). Phosphoenzyme (EP) synthesis is activated by Ca bound to the high affinity binding site located on the outer surface of vesicular membrane (Ikemoto, 1974, 1975) whereas dephosphorylation is activated by Mg^{2+} (Kanazava et al., 1971; Panet, Pick and Selinger, 1971). Yamada and Tonomura (1972) have suggested that the affinity for Ca^{2+} of the Ca^{2+} binding site was markedly reduced on phosphorylation of enzyme by ATP.

(iv) Affinity of calcium for the enzyme

Baskin and Langdon (1981) have reported that in erythrocyte membrane the Mg^{2+}-dependent ATPase activity is relatively constant while Ca^{2+}-Mg^{2+}-dependent ATPase has a complex dependence on Ca concentration. Two states, viz. low and high affinity states exist and a shift from the low to high affinity state was observed to be dependent upon Ca^{2+} and an activator protein, calmodulin (Scharff and Foder, 1978). The consequence of this shift is the stimulation of ATPase activity and enhanced Ca transformation (McIntyre and Green, 1978; Hanahan, Ekholm and Hildenbrandt 1973). On treatment of the red cell membrane with low ionic strength buffer

and EDTA, Ca^{2+} stimulated ATPase activity still associated with the membrane exhibited kinetics for one binding site for Ca.

Ikemoto (1974) recognized and purified three types of Ca binding sites which were designated as α, β, γ. Binding of Ca^{2+} at α-site activated ATP hydrolysis while binding at γ-site inhibited it. β-site appeared not to be involved in enzyme regulation. It is reported that Ca^{2+}-dependent ATPase of sarcoplasmic reticulum contains one specific high affinity site for ATP, two specific and about ten unspecific high affinity Ca binding sites (Meissner, 1973; Meissner, Conner and Fleischer, 1973).

(v) Isolation and Purification

The presence of ATPase activity other than the Na^+-K^+-ATPase in erythrocyte was shown by the evidence that ATPase activity was only partially inhibited by ouabain. Further evidence indicated that this Ca^{2+}-Mg^{2+}-ATPase activity was involved in the transport of Ca from inside of the erythrocyte to the surrounding medium (Schatzmann and Vincenzi, 1969; Weiner and Lee, 1972; Olson and Cazort, 1969; Lee and Shin, 1969). Nakeo et al., (1963) isolated two ATPases from erythrocyte membranes. The ouabain insensitive ATPase was activated by Ca^{2+} (5×10^{-4}M) as well as Mg^{2+} (5×10^{-3}M) individually and in the presence of both ions, the enzyme

activity increased additively, Another ATPase activity having 100 times lower specific activity of Ca^{2+}-Mg^{2+}-ATPase in erythrocyte membranes (Weidekamm and Brdiczka, 1975) was found in spectrin-actin fraction (Rosenthal, Kregenow and Messens, 1970; Clarke and Griffith, 1972). In the presence of both Ca^{2+} and Mg^{2+} the total enzyme activity was less than that found individually with Ca^{2+} and Mg^{2+}. The K_m was determined to be 40 uM at pH 7.0 (Wolf, 1970). Another Ca^{2+}-ATPase activity differing in kinetic properties was also detected in the membrane preparation. This enzyme had pH optimum of 8.0 at substrate concentrations ranging between 0.04 - 0.1mM. A Ca^{2+}-ATPase purified from pig erythrocyte was observed to be unstable without Ca^{2+} and an activator protein but was stabilized by Tween 20 (10 mg/ml), Triton K-100 and phospholipids. The vesicles on reconstitution catalysed a rapid ATP-dependent uptake of Ca (Haaker and Racker, 1979;). High and low affinity Ca^{2+}-Mg^{2+}-ATPase occurring together in erythrocyte membrane preparations were reported by Quist and Roufogalis (1975) but other studies (Wolf, 1972; Schatzmann, 1973) could demonstrate the existence of high affinity ATPase only.

Sarcoplasmic reticulum have been observed to contain a Ca^{2+}-stimulated ATPase (Hasselbach and Makinose, 1962; Yamamoto and Tonomura, 1967; Inesi et al., 1970; Ebashi and Lipmann, 1962). This activity was characterised by sedimentation and electrophoresis in a medium

containing Triton X-100 by McFarland and Inesi (1970). Ca pump protein from sarcoplasmic reticulum was also purified by 3 different methods (Meissner, Conner and Fleischer, 1973) were observed to account for 2/3rd of the total sacroplasmic reticulum protein and were responsible for Ca transport (Inesi, 1972; Martonosi, 1972). Sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle catalyzed an ATPase activity which required both Ca^{2+} and Mg^{2+} (Hasselbach, 1964; Weber, 1966; Ebashi and Endo, 1968). Ca^{2+}-Mg^{2+}-ATPase has been partially purified from a microsomal fraction of smooth muscle of the pig stomach (antrum) (Guytack, Schutter and Casteels, 1981).

In intestinal brush borders of chicken and rat, Ca^{2+}-dependent ATPase activity was observed to be dependent upon vitamin D (Melancon and Deluca, 1970; Martin, Melancon and Deluca, 1969). Administration of vitamin D to vitamin deficient animals markedly increased Ca^{2+}-ATPase activity.

Evidence is also forthcoming for the occurrence of Ca^{2+}-dependent ATPase activity in reproductive tissues. Shami and Redde (1971) isolated ATPase from membranes of guinea pig placenta which was preferentially activated by Ca^{2+} ions. Abla, Mroueh and Durr (1974) reported Ca^{2+}-Mg^{2+}-ATPase activity in human spermatozoa. The enzyme activity was determined over a wide range of Mg^{2+} and Ca^{2+} concentrations, separately or when present together.

Maximum activity was exhibited at 1mM Ca^{2+} and 6 mM Mg^{2+} concentrations.

Rat and cat brain is reported to contain a Mg^{2+} or Ca^{2+}-activated ATPase which was observed to be ouabain insensitive but was inhibited by sulfhydryl inhibitors, mersalyl and p-chloromercuribenzoate. A part of this activity was attributed to the presence of a contractile protein similar to actomyosin.

It is observed that Ca^{2+}-ATPase activity of smooth muscle myosin is markedly lower when compared to that of striated muscle. This has been reported for myosin isolated from chicken gizzard (Barany et al., 1966), uterus (Needham and Williams, 1963) and arteries (Gaspar-Godfroid, 1964). Purified myosin like protein isolated from the slime mold has approximately 3-times the activity of rabbit striated muscle myosin (Adelman and Taylor, 1969).

A low molecular weight, Ca^{2+}-specific ATPase distinct from dynein was shown to be present in Chlamydomonas flagella. Histochemical localization have shown this enzyme activity to be near the central microtubules and the outer dynein arms. Ca^{2+}-ATPase was stable for weeks at $_0{}^{\circ}C$ at Ca concentration of 1 to 3 times the ATP concentration. Mg inhibited this activity when added together with Ca^{2+} ions. No activity was observed when Ca^{2+} ions were replaced either with

actin + Mg^{2+} or K^{+} + EDTA. The Km for ATP was 4×10^{-4}M at Ca: ATP ratio of 2. It was reported by Watanabe and Flavin (1973) that this Ca^{2+}-ATPase does not resemble Ca transport enzymes reported from other cells/tissues.

(vI) Properties of Ca^{2+}-ATPase

(a) Nature

The Ca-transport ATPase is a highly asymmetric integral protein of the membrane. Electron microscopic pictures of freeze-fractured membranes suggest the presence of protein particles with a diameter of 90A^{o} that are mainly attached to the cytoplasmic leaflet of the membranes (Jilka, Martonosi & Tillack, 1975; Packer et al., 1974 and Malan et al. (1975). Apparently the isolation procedure does not give rise to randomised inversion of the membranes. The appearance of spindle shaped particles in electron microscope after lipid removed from membranes solubilized with deoxycholate supports the assumption that the molecule is asymmetric (Hardwicke and Green, 1974). The molecular weight of the transport unit has been estimated by gel electrophoresis of the isolated protein to be approx. 100,000 (MacFarland and Inesi, 1971; Meissner and Fleischer, 1971 and Louis and Shooter, 1972) while classical methods yield significant higher values of approx.

115,000-130,000 (Hasselbach, 1972; LeMaire, Møller and Tanford, 1976 and LeMaire et al., 1976). The notion that protein might from oligomers within the membranes presumably tetrameric units, is of considerable interest but until now is supported by rather indirect evidence (Malan et al., 1975; Martonosi et al., 1977).

(b) Optimum pH

The pH optimum of Ca^{2+}-Mg^{2+}-ATPase was reported to be between 7.0 - 7.2 in red cell membrane preparations (Schatzmann and Rossi, 1971; Schatzmann, 1975), 7.6 in skeletal muscle (McNamara, Sulakhe and Dhalla, 1971), 9.0 in brain microsomes (Nakamaru, Kosakai and Konishi, 1967) and myofibrillar tissue (Bailey, 1942). The pH optimum for activation of ATPase by Ca^{2+} lies between 8.2 and 8.5; at lower and higher pH values (7.1 and 9.5) only 50% of maximum activity was noticed (Shami and Radde, 1971). A pH optimum of 7.7 was reported by Melancon and Deluca (1970) for Ca^{2+} -ATPase found in intestinal brush border.

(c) Substrate specificity

Besides ATP, other substrates hydrolysed were ITP, GTP, CTP, UTP, acetyl phosphate, carbamyl phosphate and pNPP (in order of decreasing effectiveness). Hydrolysis rates observed ranged from 80% to less than 10% of the rate reported for ATP hydrolysis (Friedman and Makinose, 1970; Inesi, 1971; Makinose and The, 1965; Pucell and Martonosi, 1971).

(d) Ca^{2+} concentration

Ca^{2+} concentration required to activate Ca^{2+}-Mg^{2+}-ATPase activity differ with different membrane preparations. For red cell membrane Ca^{2+} concentration ranged between 300-500 μM (Dunham and Glynn, 1961), 500-700 μM (Schatzmann and Rossi, 1971) and to as low as 10-25 μM (Schatzmann, 1973; Wolf, 1970, 1972). An optimum concentration of 3-5 mM Ca^{2+} in presence of 5mM ATP (Shami and Radde, 1971) and 6 mM (Rosenthal, Kregenow and Moses, 1970) have also been reported.

(e) Mg^{2+} concentration

The requirement of Mg^{2+} for Ca^{2+}-ATPases isolated from different sources differ depending upon the source. In the erythrocytes, the ATPase activity is activated by Ca^{2+} but inhibited by Mg^{2+} (Schatzmann and Vincenzi, 1969; Rosenthal, Kregenow and Moses, 1970). The role of Mg^{2+} in relation to Ca^{2+}-ATPase activity is not understood but differs from its role in Na^{+}-K^{+}-ATPase where Mg^{2+} was found to be essential for activation (Skou, 1965). In the kidney and intestinal mucosa, Mg^{2+} stimulates the ATPase more than Ca^{2+} but either ion could replace the other. A similar metal requirement has been reported for brain ATPase which is stimulated equally well by Ca^{2+} as well as Mg^{2+} (Berl and Puszkin, 1970; Nakamaru, Kosakai and Konisai, 1967). Mg^{2+} is required for the activation of red cell

membrane ATPase by Ca^{2+} was indicated by Dunham and Glynn (1961) as well as Wins and Schoffeniels (1966 a). But Thorens (1979) observed that Mg^{2+} inhibited the maximally activated Ca^{2+}-ATPase which was interpreted to the presence of a single enzyme. Though the ionic requirement have been defined in the case of red cell membrane enzyme by Wins and Schoffeniels (1966 a), yet it is still not certain whether one or more Ca^{2+}-sensitive ATPases are present.

That the Ca^{2+} can replace Mg^{2+} in activating the ouabain insensitive component of ATPase has been shown in a number of studies (Emmelot and Bos, 1962; Taylor, 1962). Moreland and Ford (1981) obtained maximum activity at 5 mM Mg^{2+} concentration which was inhibited at lower and higher concentrations. Chiesi and Inesi (1980) have reported that Ca^{2+}-sensitive ATPase is highly sensitive to Mg^{2+} or Mn^{2+}, which produces a marked stimulation but high concentrations were observed to be inhibitory especially in the presence of low concentration of Ca^{2+}. However, this inhibition was partially prevented by Ca^{2+} suggesting a competition between two metal ions for high affinity binding site on the ATPase molecule (Inesi, Goodman and Watanabe, 1967).

Though it is generally agreed upon that Mg^{2+} is required for the dephosphorylation reaction in

sarcoplasmic reticulum (Inesi et al., 1970; Martonosi, 1969; Panet, Pick and Selinger, 1971; Kanazava et al., 1971; Makinose, 1969; Meissner, 1973) but it is recently shown by Garrahan, Rega and Alonso (1976) that Mg^{2+} may increase the rate of phosphorylation. But this matter still remains to be a point of discussion.

(f) Effect of other ions

McNamara, Sulekhe and Dhalla (1971) observed no significant difference in ATP hydrolysis in the absence and presence of 100 mM Na^+, or 100 mM K^+. But Schetzmann and Rossi (1971) reported that addition of KCl reduced the overall activity of ATPase stimulated by Ca^{2+}. In skeletal muscle, Na^+ and K^+ strongly inhibited the transfer of terminal phosphate of ATP to a protein of the sarcoplasmic reticulum in the presence of Ca^{2+}. The degree of inhibition varied with ATP concentration and temperature (DeMeis, 1972). A marked stimulation of microsomal sarcoplasmic ATPase activity by Na^+ and K^+ was observed by Hubia and Katz (1967) in presence of Ca^{2+}. Schetzmann and Vincenzi (1969) have reported that Sr^{2+} can replace Ca^{2+} but not Mg^{2+} in activating the site.

(g) Inhibitors and activators

A number of compounds have been observed to inhibit the Ca^{2+}-ATPase activity. Among these

2,4-dinitrophenol, sodium azide, oligomycin, sodium fluoride and EDTA (McNamara, Sulakhe and Dhalla, 1971), ethacrynic acid (Vincenzi, 1968), mersalyl (Schatzmann and Vincenzi, 1969; Wins and Schoffeniels, 1966 a) and PCMB (Nakamaru, Kosakai and Konishi, 1967) have been reported. Caffeine and ouabain were observed to have no effect (Schatzmann and Vincenzi, 1969).

Of the activators, a soluble protein present in hemolysate of human blood which activated Ca^{2+}-Mg^{2+}-ATPase was reported by Bond and Clough (1973). This has been purified by Luthra, Hildenbrandt and Hanahan (1976).

(h) Stability of the enzyme

Ca^{2+}-stimulated ATPase was stable without loss in enzyme activity for 2 months on keeping it at 4°C. But freezing destroyed the enzyme activity more rapidly (Shami and Radde, 1971). LeMaire, Møller and Tanford (1976) have reported that Ca^{2+}-ATPase of sarcoplasmic reticulum can exist in true solution in the presence of non-ionic detergents for several days without loss in enzyme activity.

(i) Lipid requirement of the Ca^{2+}-ATPase

Ca^{2+}-ATPase from sarcoplasmic reticulum is a classic example of an intrinsic membrane protein that is generally believed to require phospholipid for enzyme function (Martonosi, 1972). Hydrolysis of

membrane lecithin with phospholipase C results in the loss of ATPase and Ca pump activity and the two activities are restored by the addition of sonicated phospholipids (Martonosi, Donley and Halpin, 1968). Extraction of sacroplasmic reticulum with deoxycholate also leads to the inactivation of Ca^{2+}-ATPase activity (Martonosi, 1968) and cannot be activated if lipids are separated (Hardwicke and Green, 1974).

Knowless, Eyton and Recker (1976) have described a procedure for the reversible delipidation of Ca^{2+}-ATPase to a level of 5 moles of phospholipid/mole of polypeptide. Addition of lipid to delipidation preparation restored the activity to 50% of its original value. Thus, a requirement for phosphatidyl choline in the reactivation of ATPase activity was demonstrated. Ikemoto and Kasselbeck (1972) have reported that Ca^{2+}-ATPase of sarcoplasmic reticulum with modified lipid component is more sensitive to activation or inhibition by monovalent cations than the Ca^{2+}-ATPase of native sarcoplasmic reticulum membrane.

CHAPTER III

MATERIALS AND METHODS

MATERIALS AND METHODS

Adenosine 5'-triphosphate (ATP), Bovine, serum albumin (BSA), Ethyleneglycol-bis (β-amino ethyl ether) N, N'-tetraacetic (EGTA), Tris (tri-hydroxy-methyl amino methane) were the products of Sigma Chemical Co., St.Louis, U.S.A. Triton X-100 was purchased from BDH, England. Other chemicals used were of analytical grade.

METHODS

1. Collection of Semen

Semen was collected from bulls using an artificial vagina (Walton, 1945). Only those ejaculates showing high initial wave motion with a score of 2.5 to 5 (0, no motility; 5, the best motility) were used after pooling. Sperm counts were made in duplicates with a haemocytometer.

2. Washing of Semen

Semen was diluted with one volume of 0.25 M sucrose solution and centrifuged at 700 g for 10 min. The sperm cells were then washed twice with sucrose solution at 400 g for 3 min. The time and speed of centrifugation were adjusted to get a loose sperm pellet so that it could be resuspended easily on gently shaking the tubes. All washing procedures were carried out at room temperature.

3. Subcellular Fractionation

The sperms were washed three times in 0.25 M sucrose as described above. The washed sperm pellet was suspended in it, gradually cooled to 4°C and then subjected to ultrasonic vibrations at 50 watts for one minute (30 seconds at a time) in a Branson Sonifier Model B_{12}. The heds, midpieces and tails were separated by differential centrifugation at 4°C according to the method of Mohri, Mohri and Ernster (1965). The purity of isolated fractions were determined by examination with a phase contrast microscope.

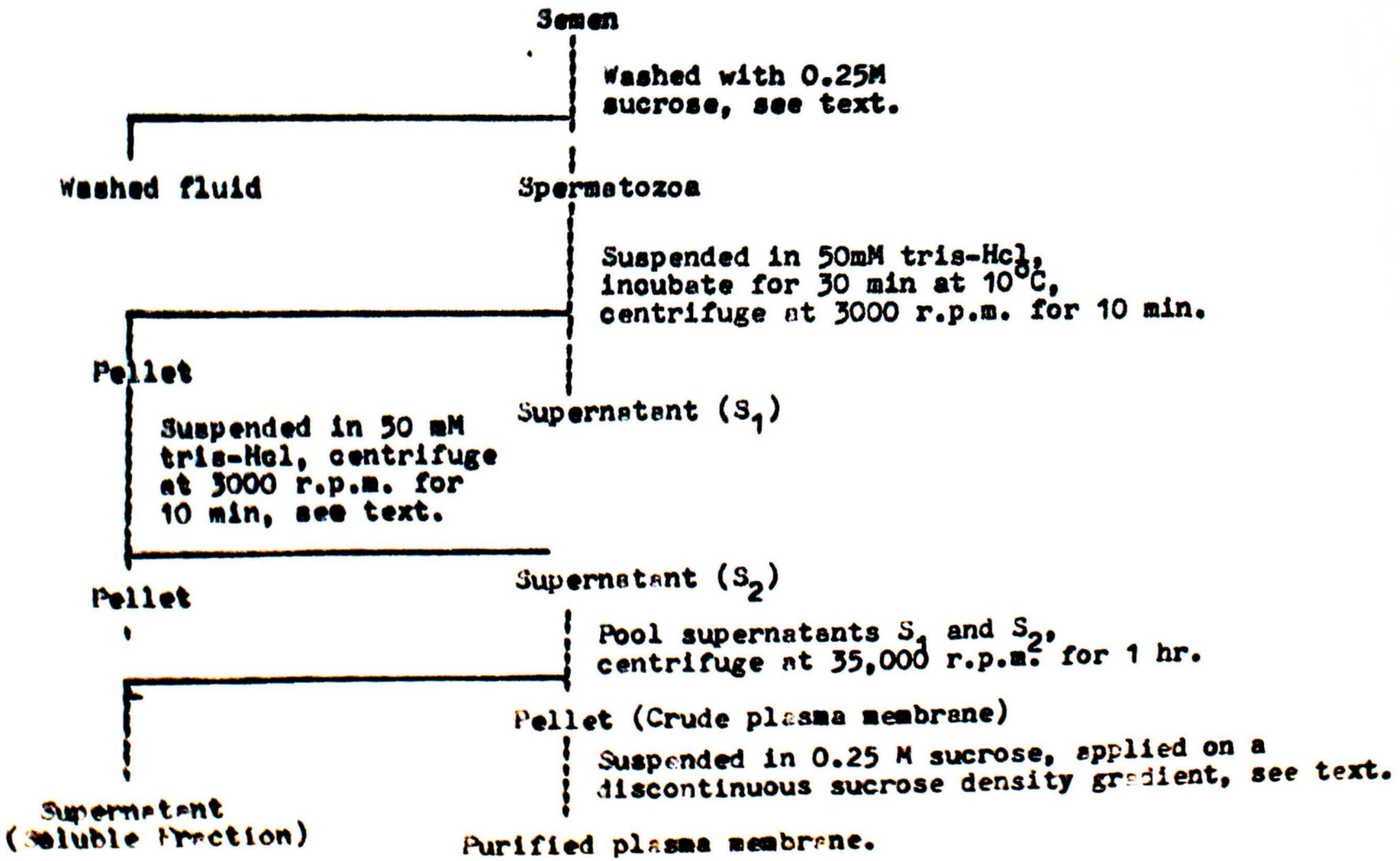
4. Triton X-100 treatment

Washed spermatozoa and sperm organelles (heads, mid pieces and tails) were treated with 0.1% Triton X-100 for 15 minutes at 37°C (Wooding, 1973). This treatment solubilizes the plasma membrane and the pellet obtained after centrifugation is residual spermatozoa/sperm organelle.

5. Isolation of Plasma Membrane

The pellet of washed spermatozoa was suspended in the hypotonic buffer (50 mM tris, pH 7.5) and the sperm concentration was adjusted from 1 x 10^5 to 1 x 10^6 spermatozoa/ml. The tube containing 30 to 40 ml of sperm suspension was placed in a beaker containing water and was then placed in a cold room (4 to 5°C)

Flow sheet for the preparation of plasma membrane



for a period of 30 min to obtain the temperature of sperm suspension to 4°C.

The suspension was centrifuged at 4°C at 3,000 r.p.m. for 10 min. The supernatant (S_1) was removed and the pellet was washed with the same volume of tris-HCl buffer as of the original suspension and supernatant (S_2) was obtained. The two supernatants (S_1 and S_2) were then pooled and centrifuged in a Beckman (Model L) Centrifuge at 35,000 r.p.m. for 1 hr. The supernatant was discarded and the pellet was suspended in a small volume of 0.25M sucrose and termed the "crude plasma membrane". It was then purified in a discontinuous sucrose density gradient.

6. Purification of sperm plasma membrane

A discontinuous sucrose gradient having densities of 1.45M, 1.35M, 1.25M, 1.15M and 0.25M was prepared; the total volume being 5 ml. The crude plasma membrane preparation (3-4 mg protein) in 0.25M sucrose was layered on top of the gradient. The tubes were spun in a swinging bucket rotor (SW 50.1) at 30,000 r.p.m. for 3 hr. The plasma membrane was obtained as a single band at the interphase of 1.25M and 1.15M sucrose.

7. Enzyme assay

Ca^{2+}-dependent ATPase activity was determined by estimating the release of inorganic phosphorus from ATP. The assay mixture contained 3mM ATP, 50mM tris-HCl

[illegible]

2. Analytical methods

(1) Phosphorus estimation

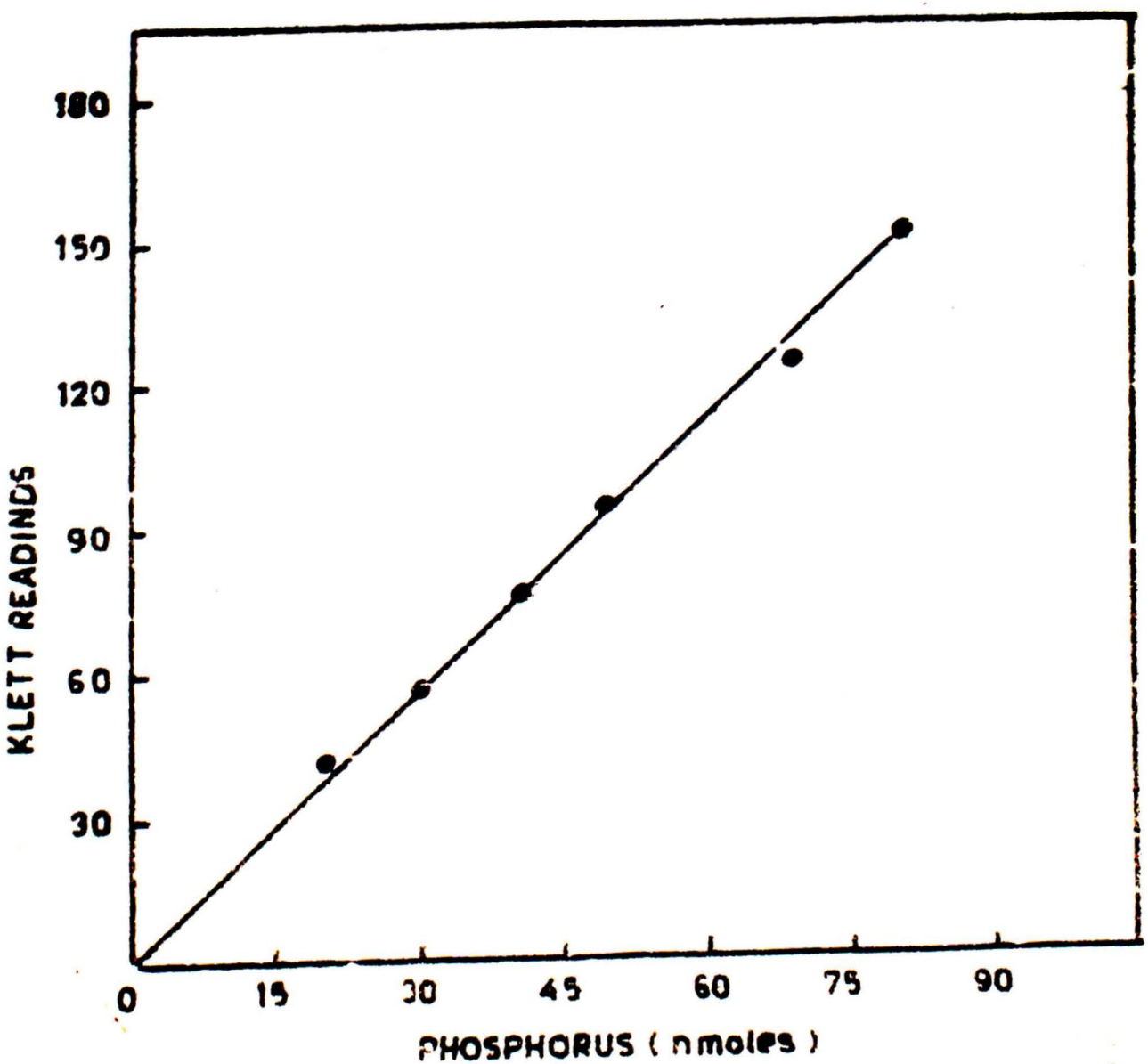
Phosphorus was estimated by the method of Fiske, Subbarow and Ogura (1925). To the sample taken in a 100 c.c. flask was added 2.0 ml of 1 N H$_2$SO$_4$ and 1 ml of 10% ammonium molybdate and 2% ammonium chloride and the mixture made up to the mark with distilled water. The blue colour developed by the addition of 0.4 ml of 1 N amino naphthol sulphonic acid was read in a Klett-Summerson photoelectric colorimeter with a filter having maximum transmission at 660 mμ. A blank was run for each sample in which 1 ml of 10% trichloroacetic acid was added before the addition of the enzyme. The blank was subtracted from the sample reading. The inorganic phosphorus in the mixture was estimated as described below.

A unit of enzyme activity was defined as the amount of P$_i$ liberated in 30 min at 37°C, while the specific activity was defined as units per mg of protein.

(ii) Protein estimation

Proteins were estimated by the method of Lowry et al. (1951) with bovine serum albumin (Armour) as the standard.

Fig.1 Standard curve for the estimation of phosphorus by the method of Chen, Toribara and Huber (1956).


180
150
120
90
60
30
KLETT READINGS
0
15
30
45
60
75
90
PHOSPHORUS (n moles)

CHAPTER IV

RESULTS

RESULTS

1. Intracellular distribution of Ca^{2+}-ATPase in bull sperm

The results in Table I show that Ca^{2+}-ATPase is distributed in the head, midpiece and tail fractions of bull spermatozoa. The recovery of total enzyme activity was 90% and protein 92% relative to the intact sperm. An increase of 3.6% in enzyme activity was observed on sonication of sperm suspension. The head fraction had 16.5%, midpieces 20.0%, tails 2.0% and 51.5% Ca^{2+}-ATPase activity was solubilized. The combined activity of midpiece + tail fractions was 22% of the total activity present in the sonicated suspension.

2. Solubilization of Ca^{2+}-ATPase from sperm organelles

Head, mid piece and tail fractions were treated separately with Triton X-100 to remove the plasma membrane. All the enzyme activity present in head was solubilized and the head pellet showed no activity. No midpiece fraction, about 56% of the total activity was solubilized on Triton X-100 treatment while 42% was with the pellet. The total recovery of enzyme activity was 98.4%. In tail fraction, 66.2% Ca^{2+}-ATPase activity was recovered in soluble form and 53% bound with the pellet with a total recovery of 119% (Table II).

Table I. Distribution of Ca^{2+}-ATPase in bull spermatozoa.

Fraction	Protein (mg)	Total Activity	Specific Activity	Activity relative to intact sperm
Sperm suspension	24.60	19.07	0.77	100
Sonicate	24.60	19.76	0.80	103.6
Head	12.04	3.16	0.26	16.5
Mid piece	7.33	3.82	0.52	20.0
Tail	2.31	0.38	0.16	2.0
Supernatant	0.97	9.82	10.14	51.5

Enzyme activity is expressed in terms of units whereas a unit of enzyme activity is the μ moles of phosphorus liberated in 30 min at 37°C.

Table II. Solubilization of Ca^{2+}-ATPase from bull sperm heads, mid pieces and tails.

Fraction	Activity	Specific Activity	% Distribution
Heads	0.91	0.28	100.0
(i) Pellet	0.00	0.00	0.0
(ii) Supernatant	0.15	0.23	16.4
Mid piece	2.66	1.37	100.0
(i) Pellet	1.14	0.51	42.8
(ii) Supernatant	1.48	1.84	55.6
Tails	2.46	2.11	100.0
(i) Pellet	1.30	1.87	52.8
(ii) Supernatant	1.63	0.76	66.2

Enzyme activity is expressed in terms of units whereas a unit of enzyme activity is the n moles of phosphorus liberated in 30 min at 37°C.

These results would show that Ca^{2+}-ATPase is not exclusively localized in sperm plasma membrane.

3. Ca^{2+}-ATPase activity in bull sperm plasma membrane

Bull sperm plasma membrane was prepared by subjecting the washed spermatozoa to hypotonic shock in 50 mM tris-Hcl buffer, pH 7.4 at refrigerated temperature. The crude plasma membrane was centrifuged and further purified on sucrose density gradient. The distribution of Ca^{2+}-ATPase activity showed 61.9% recovery of total enzyme activity on hypotonic treatment with 41.3% in residual sperm and 19.7% in soluble fraction. The data during the preparation of plasma membrane is given in Table III.

Plasma membrane from washed bull spermatozoa was also removed by treatment with Triton X-100. This method gave a recovery of 110% with 65% activity in the residual spermatozoa and 45% in the soluble fraction.

4. Properties of Ca^{2+}-ATPase in purified plasma membrane and residual sperm

(i) Effect of enzyme concentration

The effect of varying the enzyme concentration over a 7 fold range for purified plasma membrane and 10 fold for demembranated spermatozoa is shown in Figs. II and III. The reaction rates were observed to be linear in both cases; upto 6 fold protein concentration for purified plasma membrane and 5 fold protein concentration for demembranated spermatozoa.

Table III. Isolation of bull sperm plasma membrane by hypotonic treatment (Method I) and Triton X-100 treatment (Method II) and the distribution of Ca^{2+}-ATPase.

Method	Fraction	Protein (mg)	Activity	Specific Activity	Activity (%)
I	Whole sperm suspension	10.12	16.22	1.60	100.0
	Demembranated sperm	8.15	6.71	0.82	41.3
	Supernatant	2.44	2.06	0.84	12.7
	Crude plasma membrane	0.19	1.14	5.81	7.0
	Purified plasma membrane	0.14	0.73	5.02	4.5
II	Whole sperm suspension	10.12	16.22	1.60	100.0
	Demembranated sperm	7.13	10.57	1.48	65.1
	Supernatant	3.18	7.42	2.33	45.7

Enzyme activity is expressed in terms of units whereas a unit of enzyme activity is the μ moles of phosphorus liberated in 30 min at 37°C.

Fig.2 Effect of enzymes concentration on the activity of Ca^{2+}-ATPase in plasma membrane.

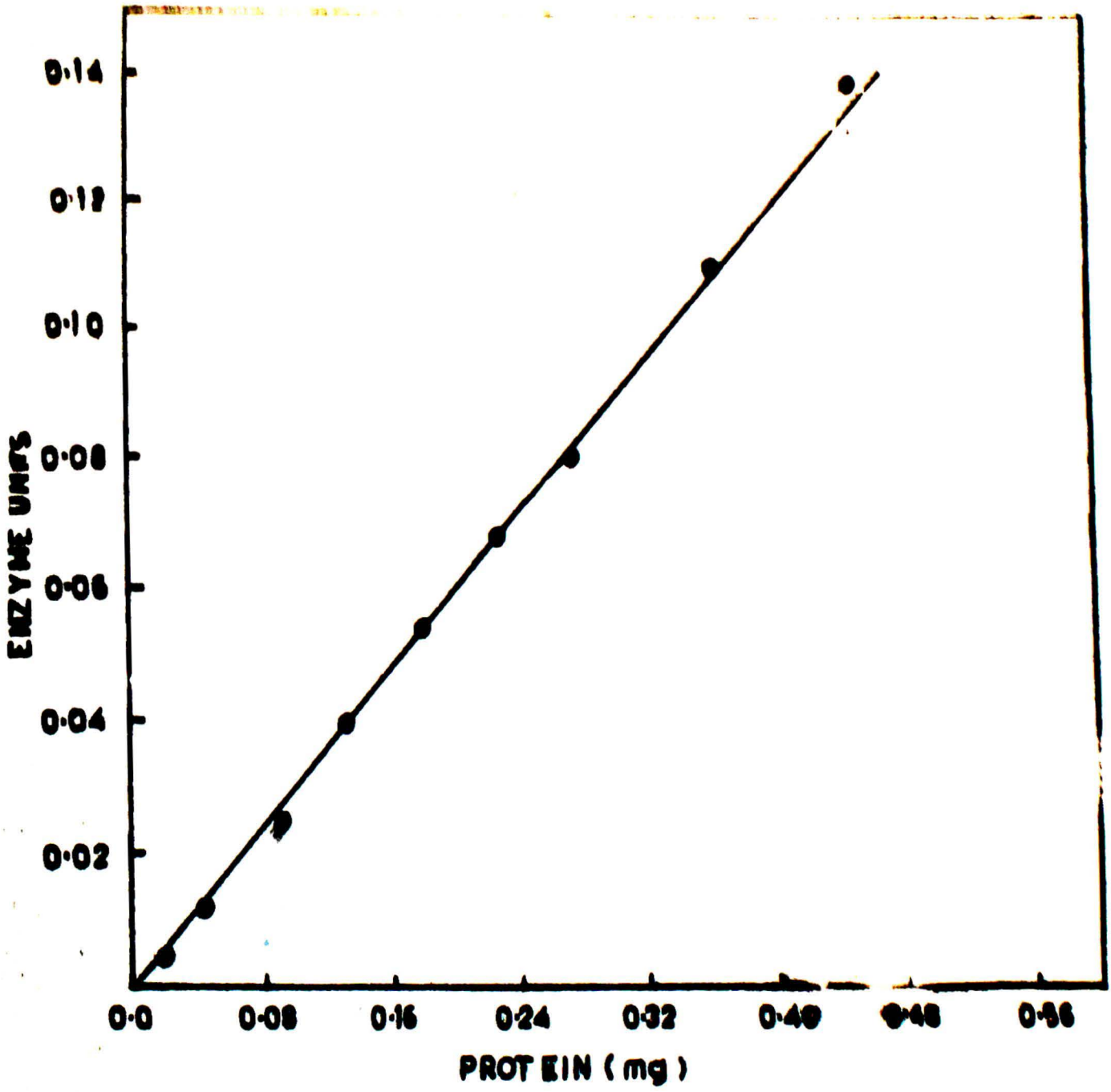
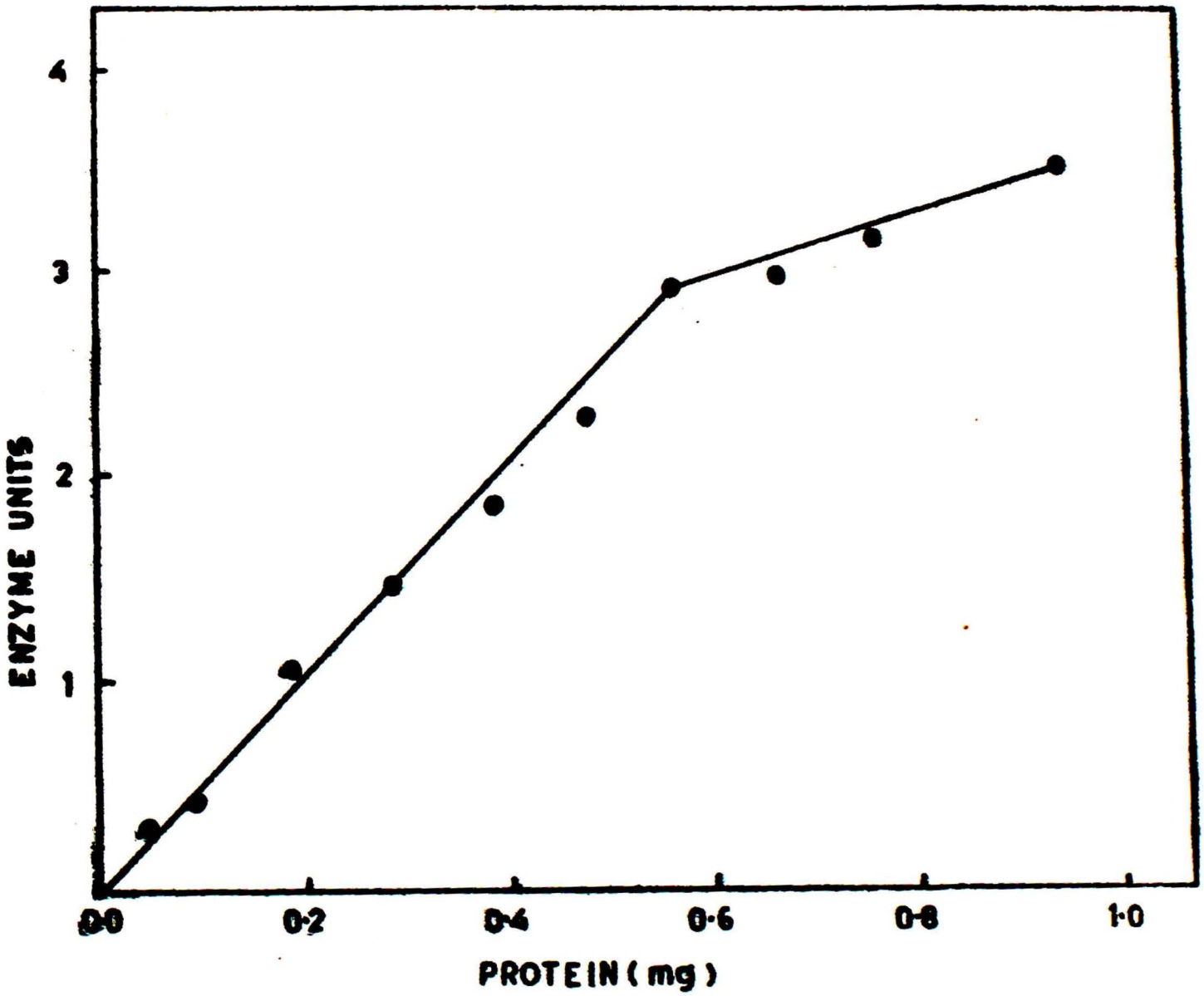

0·14
0·12
0·10
0·08
0·06
0·04
0·02
ENZYME UNITS
0·0
0·08
0·16
0·24
0·32
0·40
0·48
0·56
PROTEIN (mg)

Fig. 1 Effect of enzyme concentration on the activity of Ca^{2+}-ATPase in residual sperms.


ENZYME UNITS
4
3
2
1
0·0
0·2
0·4
0·6
0·8
1·0
PROTEIN (mg)

(ii) Effect of incubation period

The rate of hydrolysis of ATP by purified plasma membrane in the presence of Ca^{2+} ions was linear for 30 min but decreased thereafter (Fig. IV). Likewise, the rate of hydrolysis of ATP by demembranated sperm in presence of Ca^{2+} ions was linear for 40 min (Fig. V) and levelled off afterwards.

(iii) Effect of pH

pH optimum of Ca^{2+} ATPase of purified plasma membrane as well as of demembranated sperm was determined between pH 5.5 to 10.0 using the buffers Tris-Maleate (pH 5.5 - 7.0), Tris HCl (pH 7.5 - 9.0) and Glycine-NaOH (pH 9.5 - 10). The pH of the solutions were checked before and after the enzymatic assay for constancy of pH values. During various runs for optimal pH, pH optima of 8.5 (Fig. VI) for purified plasma membrane and 9.0 for demembranated spermatozoa (Fig. VII) was obtained. For purified plasma membrane, no activity was observed at pH 5.5 while at pH 10.0, 44.4% of maximum activity was noticed. In the case of demembranated spermatozoa, a broad peak of activity was obtained between pH 6.5 - 7.0 in addition to the sharp peak at pH 9.0.

(iv) Effect of substrate concentration

Ca^{2+}-ATPase of purified plasma membrane as well as of demembranated sperm gave typical biphasic

Fig.4 Effect of incubation period on the activity of Ca^{2+}-ATPase in plasma membrane.

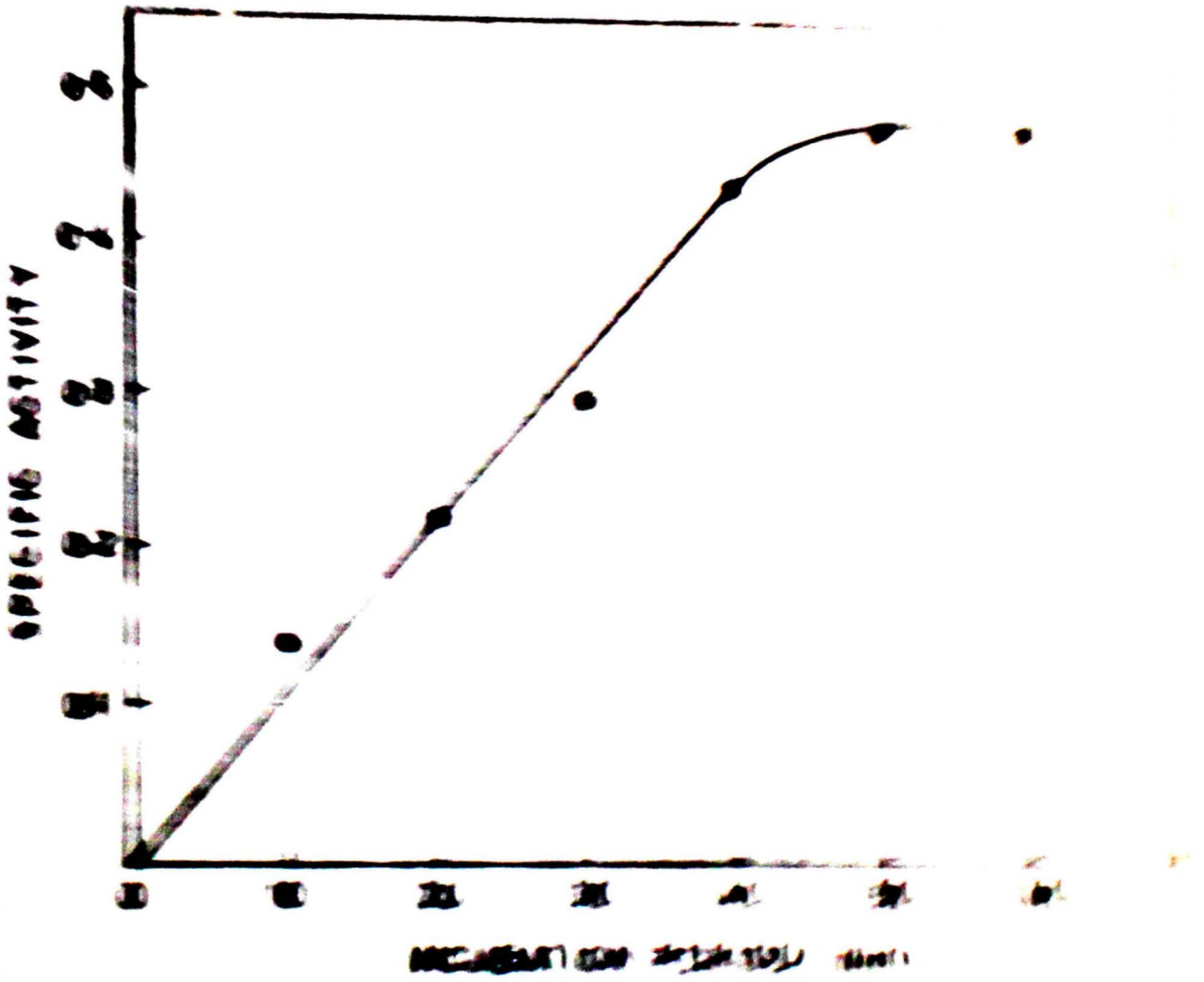


Fig.6 Effect of pH on the activity of Ca^{2+}-ATPase in plasma membrane.

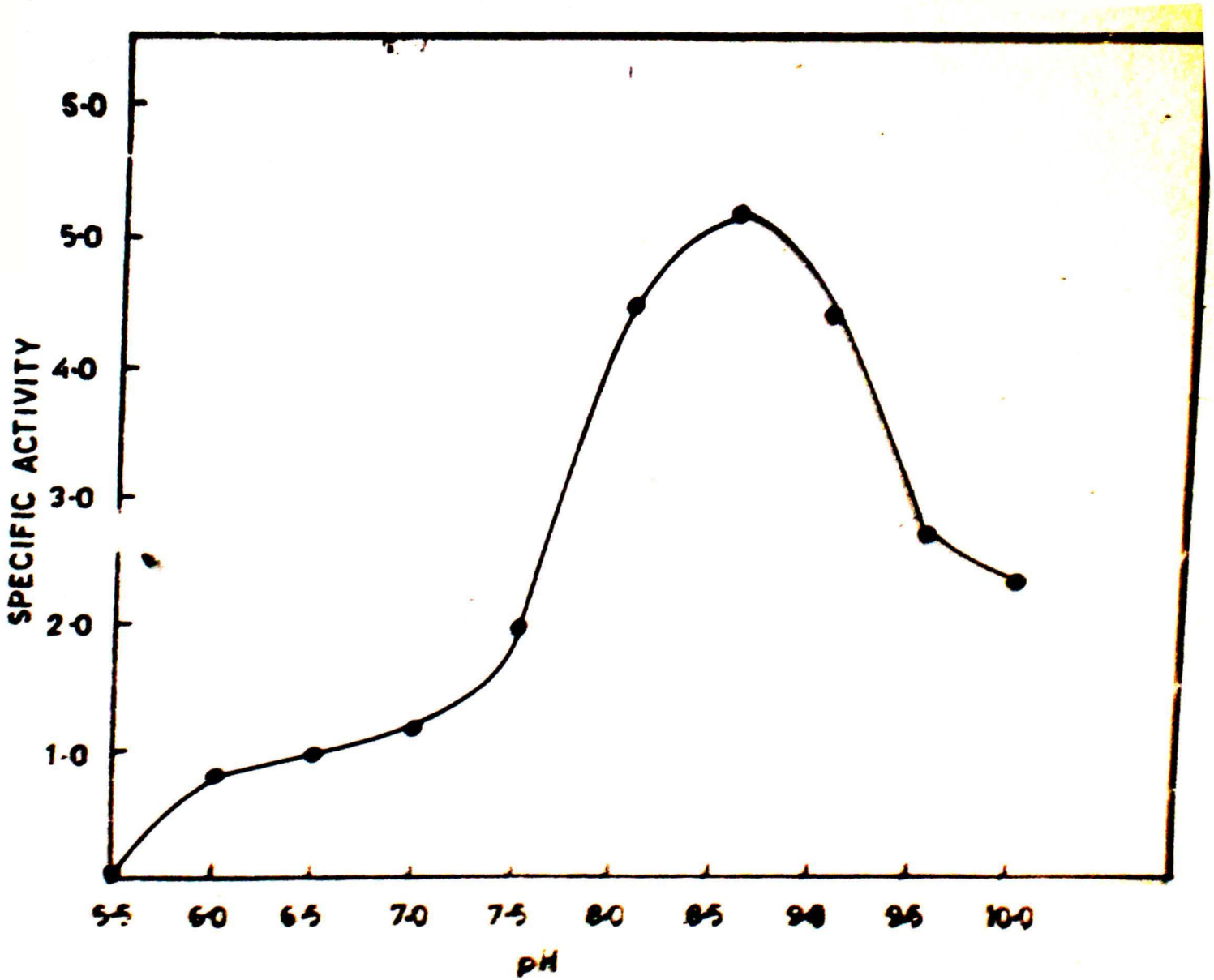

SPECIFIC ACTIVITY
5·0
4·0
3·0
2·0
1·0
5·5
6·0
6·5
7·0
7·5
8·0
8·5
9·0
9·5
10·0
pH

Fig.7 Effect of pH on the activity of Ca^{2+}-ATPase in residual sperms.

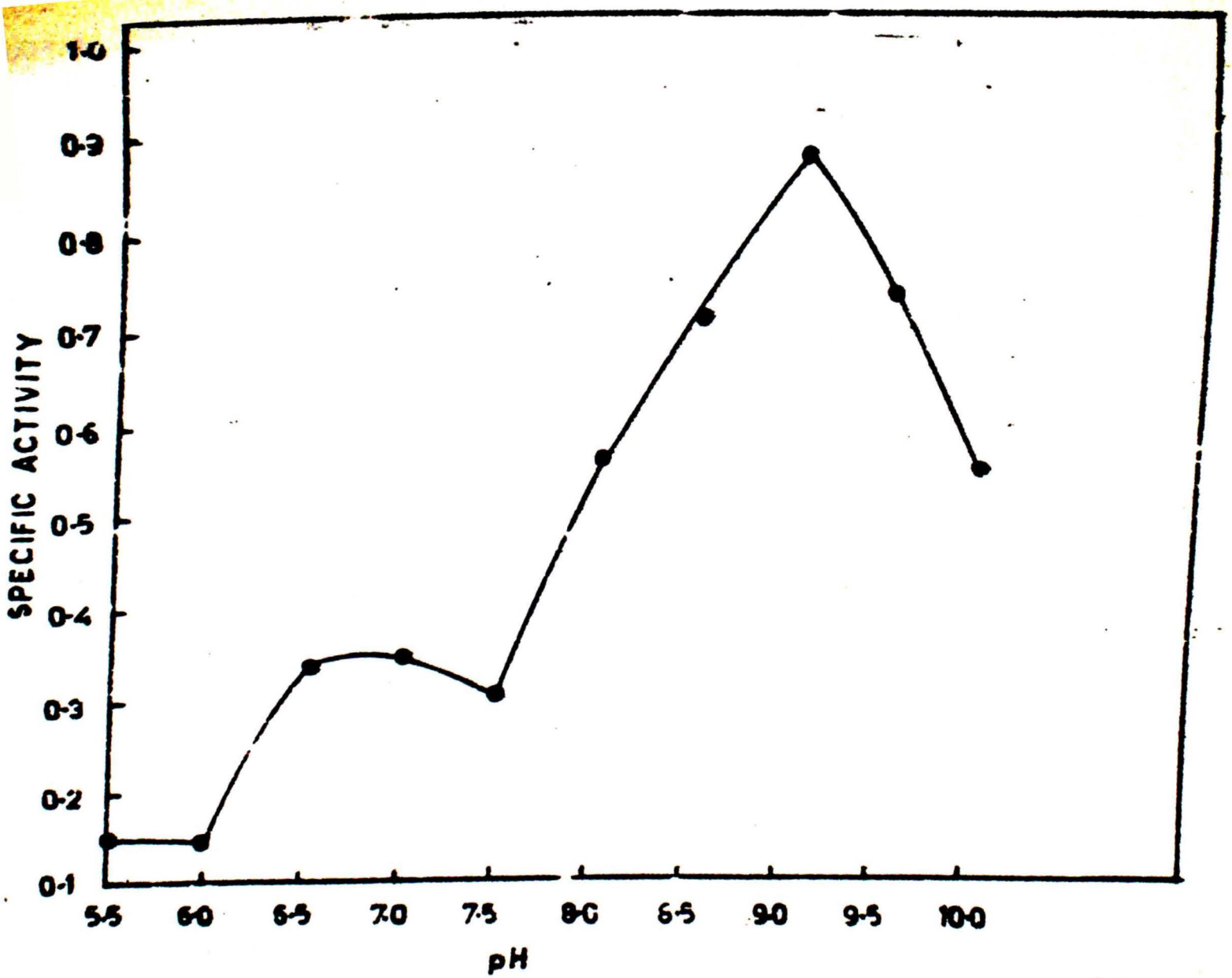

SPECIFIC ACTIVITY
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5·5
6·0
6·5
7·0
7·5
8·0
8·5
9·0
9·5
10·0
pH

Fig.8 Double reciprocal plot of Ca^{2+}-ATPase activity in plasma membrane as a function of ATP concentration.

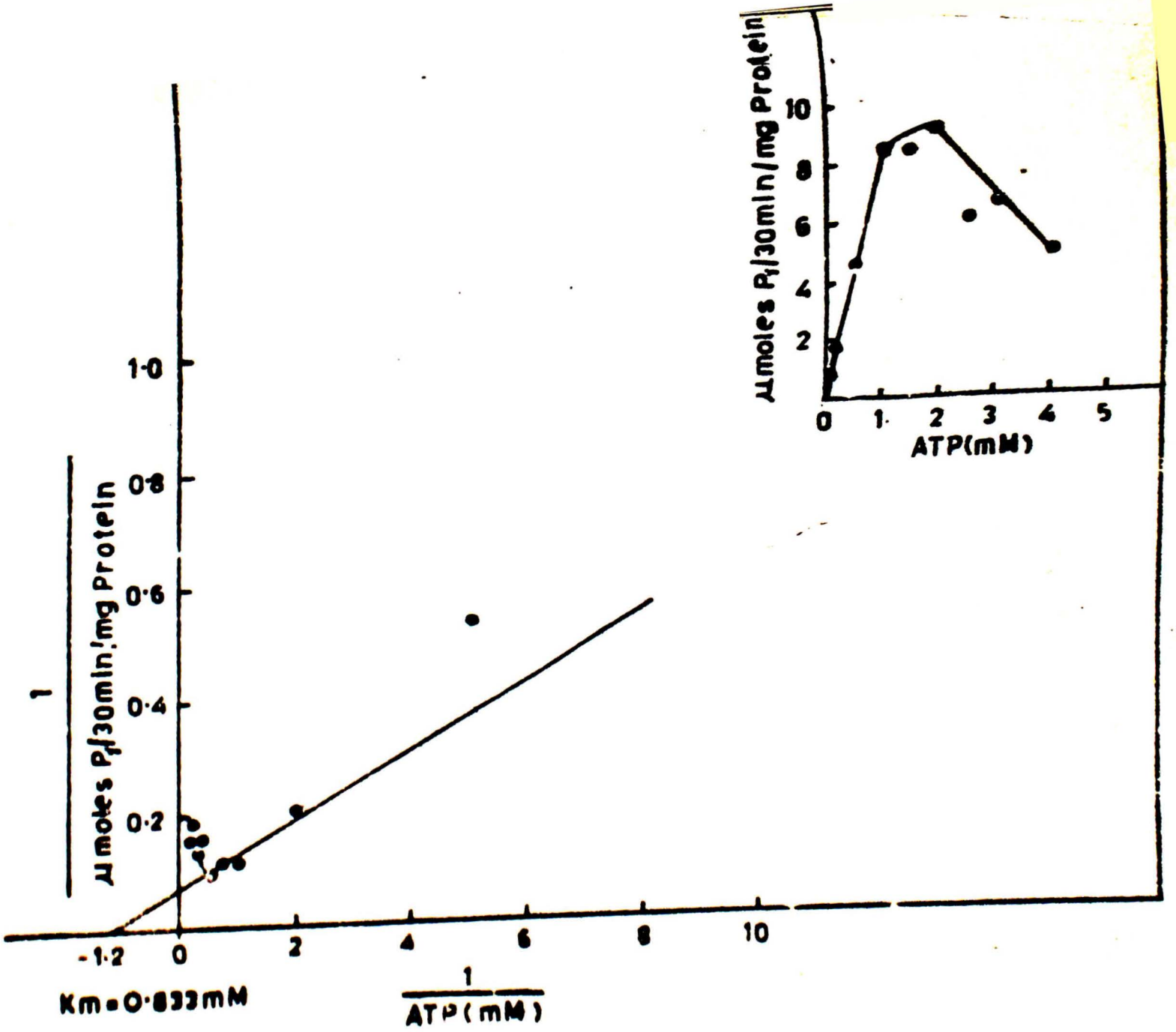
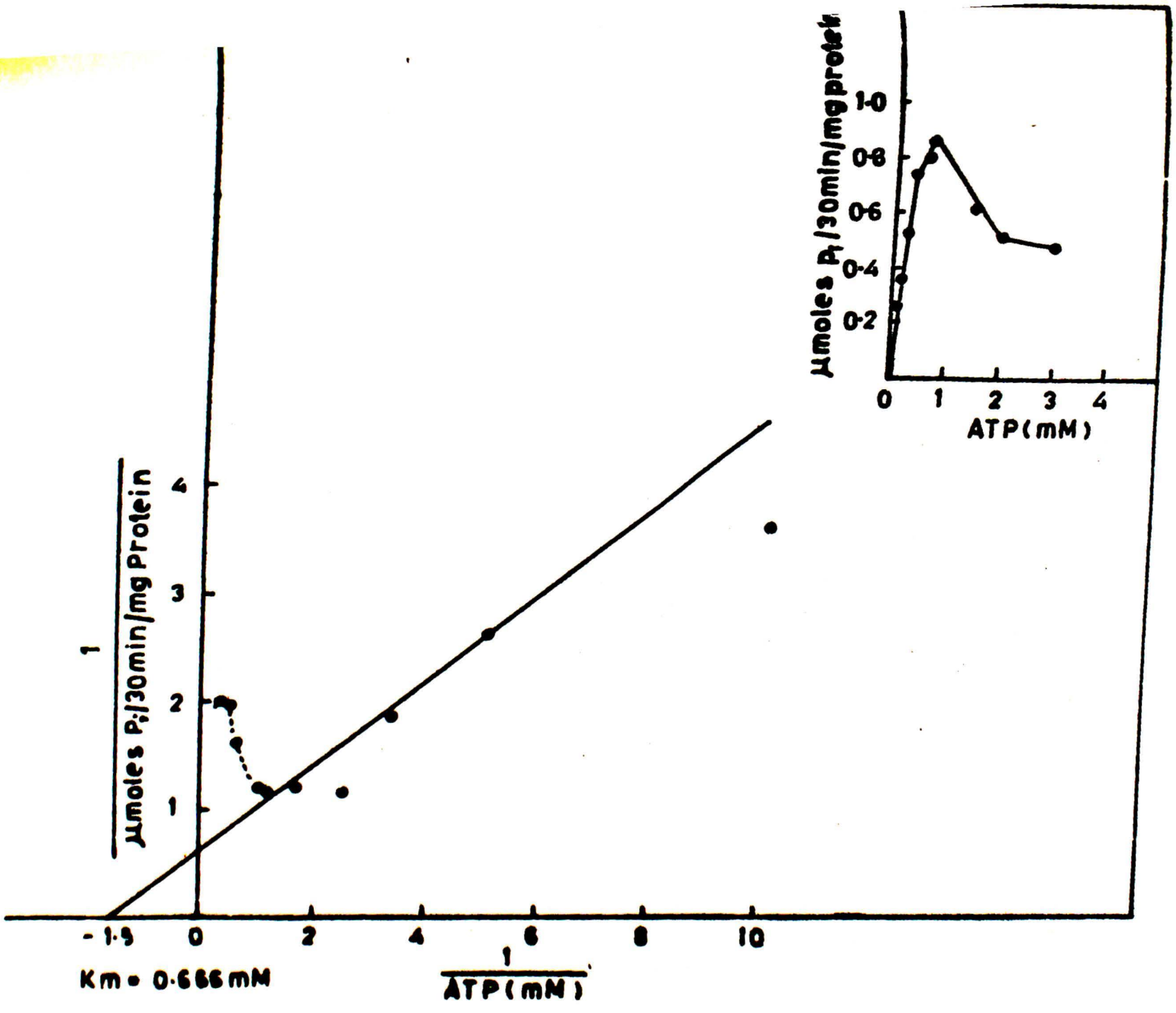

µmoles Pi/30min/mg Protein
10
8
6
4
2
0
1
2
3
4
5
ATP(mM)
1
µmoles Pi/30min/mg Protein
1·0
0·8
0·6
0·4
0·2
-1·2
0
2
4
6
8
10
Km = 0·833mM
1
ATP (mM)

Fig.9 Double reciprocal plot of Ca^{2+}-ATPase activity in residual sperms as a function of ATP concentration.


µmoles Pi/30min/mg protein
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
ATP(mM)
1
µmoles Pi/30min/mg Protein
4
3
2
1
-1.9
0
2
4
6
8
10
Km = 0.666 mM
1
ATP(mM)

kinetics (Figs. VIII and IX). The reaction rate was of first order upto 1 mM substrate concentration both for plasma membrane as well as demembranated sperm Ca^{2+}-ATPase.

The Km values of Ca^{2+}-ATPases were calculated by using the data from Figs. VIII and IX and representing it graphically according to the method of Double Reciprocal Plot. The dissociation of the enzyme substrate complex (Km) for purified plasma membrane was 0.83mM and that for demembranated sperm 0.66mM. The Vmax for the purified plasma membrane and demembranated sperm was 9.45 u moles of Pi/30 min/mg protein and 0.87 u moles of P_i/30 min/mg protein.

-.-.-.-.

CHAPTER V

DISCUSSION

DISCUSSION

In two sperm functions viz., motility and fertilizing ability, evidence is available about the involvement of calcium. A direct relationship between calcium uptake and motility activation was observed by Babcock, First and Lardy (1976). In presence of agents which alter membrane permeability, two influxes of calcium into spermatozoa were recorded. The influx which sequester Ca^{2+} ions in the mitochondria did not affect either the respiratory or kinetic activity but the accumulation of Ca^{2+} in the extra mitochondrial region resulted in the activation of sperm motility. A requirement of Ca^{2+} to maintain the motility of hamster spermatozoa had been discribed earlier by Morita and Chang (1970) who failed to observe similar requirement for guinea pig, rat and rabbit spermatozoa. But subsequent studies showed the involvement of Ca^{2+} for motility of rat (Davies, 1978) and guinea pig (Morton et al., 1974; Hyne and Garbers, 1979; Singh, Babcock and Lardy, 1978).

The involvement of Ca^{2+} in capacitation, acrosome reaction and fusion processes is a subject of great current interest. Together, these processes constitute the fertilization process. Bovine and guinea pig epididymal spermatozoa were observed to

accumulate calcium during incubation *in vitro* which was stimulated in the presence of phosphate. In contrast, washed ejaculated bovine spermatozoa were incapable of accumulating exogenously supplied Ca^{2+}. A smaller molecular weight protein of minor abundance was isolated from bovine seminal plasma and has been characterized (Singh, 1980). Spermatozoa on coming into contact with accessory gland secretion at ejaculation has this protein added on to its surface which makes the sperm plasma membrane impermeable to calcium ions. In the female seproductive tract, it prevents or delays the uptake of Ca^{2+} ions until the time this component is removed from the sperm surface. Singh, Babcock and Lardy (1978) observed that guinea pig spermatozoa incubated in a minimal incubation medium has two influxes of Ca^{2+} ions. The initial uptake was apparently unrelated to capacitation but is associated with spermatozoal surface as revealed by ^{45}Ca uptake experiments. The secondary uptake of Ca^{2+} was observed during incubation under conditions that produce capacitation *in vitro* and the time course of this paralleled that of acrosome reaction.

These studies thus clearly establish the presence in sperm organelles of transport systems for influxes of Ca^{2+} observed both the motility activation

as well as during fertilization. In the case of red blood cells it is known that Ca^{2+} is transported across the membrane through ATPase which is Ca^{2+}-dependent and is distinct from Na^{+}-K^{+}-ATPase associated with plasma membrane. (Schatzmann and Vincenzi, 1969; Weiner and Lee, 1972; Olson and Cazort, 1969; Lee and Shin, 1969). Vijayasarathy, Shivaji and Balaram (1980) have detected the presence of Ca^{2+}-ATPase in bull spermatozoa and have reported its occurence exclusively with plasma membrane while Abla, Mrouch and Durr (1974) have described Ca^{2+}-ATPase in human sperm. Intracellular distribution of Ca^{2+}-ATPase, in the present study, has revealed 16.5% of enzyme activity to be associated with sperm head, 20% with mid piece, 2% with tail and 51.5% was solubilized. Removal of plasma membrane around the sperm organelles (Table II) with Triton X-100 revealed that Ca^{2+}-ATPase is exclusively not localized in sperm plasma membrane. The mid piece fraction, on treatment with Triton X-100, had retained 42% of total activity with the mitochondria. While in the head most of the activity was solubilized and in the tail Ca^{2+}-ATPase was still associated with microtubule fraction. These results were confirmed when plasma membrane was prepared by two methods viz. by subjecting the washed spermatozoa to hypotonic shock in 50mM tris-Hcl buffer and by treatment with Triton X-100 In both the methods, the Ca^{2+}-ATPase was found to be

associated with demembranated sperm tissue (1 hr 111).
The plasma membrane was purified by [illegible] density gradient and a comparison was made between the properties of Ca^{2+}-ATPase of purified plasma membrane and that still associated with demembranated spermatozoa.

Purified plasma membrane enzyme had a pH optimum of 8.5 while that of demembranated spermatozoa showed activity which was optimum at pH 9.0. The two enzymes also differed in their [illegible] purified plasma membrane enzyme [illegible] spers 0.6 mM. Thus, the preparation of the enzyme studied would show the presence of more than one Ca^{2+}-ATPase in their membranes. It would be of interest in the future studies to investigate the role of calmodulin in Ca^{2+}-ATPase detected in the sperm tail membrane [illegible] mid piece and tail, in the development of [illegible] were theorized to play an important role in [illegible] activation and release of [illegible].

CHAPTER VI

SUMMARY

SUMMARY

1. Ca^{2+}-ATPase was assayed in bull spermatozoa and intracellular distribution revealed 16.5% enzyme activity in head, 20% in mid piece, 2% in tail and 51.5% in soluble supernatant.
2. Treatment of different sperm organelles (heads, mid pieces and tails) with Triton X-100 solubilized 100% activity in sperm heads, 56% in mid pieces and 66.2% in tails. In the whole spermatozoa, treatment with Triton X-100 solubilized 45% Ca^{2+}-ATPase activity compared to 62% obtained by subjecting the spermatozoa to hypotonic shock.
3. Plasma membrane prepared by hypotonic shock was further purified by discontinuous sucrose gradient and the properties of Ca^{2+}-ATPase were compared with thet of the enzyme in demembrenated spermatozoa.
4. Ca^{2+}-ATPase activity of purified plasma membrane was linear upto 6 fold protein concentration and for 30 min of incubation at 37°C. The maximum activity was observed at pH 8.0. The Km was 0.05mM with Vmax of 9.45 µ moles of P_i/30min/mg protein.

5. Ca^{2+}-ATPase activity in [illegible] showed a linear relationship with [illegible] concentration and upto [illegible] at 37°C. The pH optimum was [illegible] 9.0 with a minor peak between pH [illegible]. The Km value was determined by the [illegible] Vmax of 0.87 [illegible]

6. The presence of [illegible] bull spermatozoa [illegible] the transport of [illegible] activation [illegible] is under [illegible]

BIBLIOGRAPHY

BIBLIOGRAPHY

Able, [illegible] [illegible] cation-dependent [illegible] J.Reprod. Fert., [illegible]

Adelman, [illegible] and [illegible] [illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

Brierley, G.P., Murer, E. and Green, D.E. (1963) Participation of an intermediate of oxidative phosphorylation in ion accumulation by mitochondria. Science, 140: 60-62.

Carsten, M.E. (1969) Role of calcium binding of sarcoplasmic reticulum in the contraction and relaxation of uterine muscle. J. Gen. Physiol., 53: 414-426.

[illegible], Y.J., [illegible] and [illegible] (1971) Active uptake of Ca^{2+} and Sr^{2+}-activated Mg^{2+}-ATPase in rat cell membrane fragments. J. Gen. Physiol., 57: 202-219.

[illegible], P.J. Jr., Barbara, T.C. and [illegible] (1968) Microdetermination of phosphorus. Anal. Chem., 28: 1756-1758.

[illegible], M. and [illegible] (1971) Magnesium (2-), and manganese oxidation of calcium (2-), transport and ATPase activity in sarcoplasmic reticulum. Biochemistry, 10: 2312-2318.

[illegible], M., and Griffith, J. (1972) Isolation and properties of a protein from human erythrocyte membranes. J. Cell. Biol., 52: 642(abstr.)

[illegible], R.A. (1968) Effect of calcium on motility and fertilization by sea urchin [illegible] Exp. Cell. Res. Suppl. Bull. Cell. Res., 12: [illegible]

[illegible], L. (1972) Phosphorylation of the membrane protein of the sarcoplasmic reticulum: inhibition by low Ca^{2+}. Biochemistry, 11: 2466-2469.

[illegible], E.F. and Hynes, [illegible] (1969) Ultrastructural [illegible] and the active movements of chick embryo lens. J. Physiol. (London), 204: 27-37.

Ebashi, S., and Endo, M. (1968) Calcium ion and muscle contraction.
Progr. Biophys. Mol. Biol., 18: 123-183

Ebashi, S., and Lipmann, F. (1962) Adenosine triphosphatase-linked concentration of calcium ions in a particulate fraction of rabbit muscle.
J.Cell. Biol., 14: 389-400.

Emmelot, P. and Bos, C.J. (1962) Adenosine triphosphatase in the cell-membrane fraction from rat liver.
Biochim. Biophys. Acta, 58: 374-375.

Fitzpatrick, D.F., Landon, E.J., Debbas, G. and Hurwitz, L. (1972) A calcium pump in vascular smooth muscle.
Science, 176: 305-306.

Friedman, Z. and Makinose, M. (1970) Phosphorylation of skeletal muscle microsomes by acetylphosphate.
FEBS Lett., 11: 69-72.

Garrahan, P.J., Rega, A.F. and Alonso, G.L. (1976) The interaction of magnesium ions with the calcium pump of sarcoplasmic reticulum.
Biochim. Biophys. Act, 448: 121-132.

Gaspar-Godfroid, A. (1964) Adenosinetriphosphatase activity of tomomyosin of bovine carotids.
Angiologica, 1: 12-35.

Godfraind, T., Sturbois, X. and Verbeke, N. (1976) Calcium incorporation by smooth muscle microsomes.
Biochim. Biophys. Act., 455: 254-268.

Hasker, H. and Hacker, A. (1979) Purification and reconstitution of the Ca^{2+}-ATPase from plasma membranes of pig erythrocytes.
J.Biol. Chem., 254: 6598-6602.

Hanahan, D.J., Ekholm, J. and Hildenbrandt, G. (1973) Biochemical variability of human erythrocyte membrane preparations, as demonstrated by sodium-potassium-magnesium and calcium adenosine triphosphatase activities. Biochemistry, 12: 1374-1387.

Hardwicke, P.M.D. and Green, N.M. (1974) The effect of delipidation on the adenosine triphosphatase of sarcoplasmic reticulum. Electron microscopy and physical properties. Eur. J.Biochem., 42: 182-193.

Hasselbach, W. (1964) Relaxing factor and the relaxation of muscle. Prog. Biophys. Mol. Biol., 14: 167-222.

Hasselbach, W. (1972) Molecular Bioenergetics and Macromolecular Biochemistry, pp 149-171. Springer Verlag, Berlin.

Hasselbach, W. (1978) The reversibility of the sarcoplasmic calcium pump. Biochim. Biophys. Acta, 515: 23-53.

Hasselbach, W. and Makinose, M. (1961) Die calcium pumpe der "leichten" gallen der muskulatur und ihre abhängigkeit von der adenosintriphosphat-spaltung. Biochem. Z., 333: 518.

Hasselbach, W. and Makinose, M. (1962). ATP and active transport. Biochem. Biophys. Res. Commun., 7: 132-136.

Hasselbach, W. and Makinose, M. (1963) Über den mechanismus des calcium-transportes durch die membranen des sarkoplasmatischen reticulums. Biochem. Z., 339: 94-111.

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

Ikemoto, N. (1974) The calcium binding sites involved in the regulation of the purified adenosine triphosphatase of the sarcoplasmic reticulum.
J.Biol. Chem., 249: 649-651.

Ikemoto, N. (1975) Transport and inhibitory Ca^{2+}-binding sites on the ATPase enzyme isolated from the sarcoplasmic reticulum.
J.Biol. Chem., 250: 7219-7224.

Inesi, G. (1971) p-nitrophenyl phosphate hydrolysis and calcium ion transport in fragmented sarcoplasmic reticulum.
Science, 171: 901-903.

Inesi, G. (1972) Active transport of calcium ions in sarcoplasmic membranes.
Ann. Rev. Biophys. Bioeng., 1: 191-210.

Inesi, G., Goodman, J. and Watanabe, S. (1967) Effect of diethyl ether on the adenosine triphosphatase activity and the calcium uptake of fragmented sarcoplasmic reticulum of rabbit skeletal muscle.
J.Biol. Chem., 242: 4637-4643.

Inesi, G., Maring, E., Murphy, A.J. and McFarland, B.H. (1970) A study of the phosphorylated intermediate of sarcoplasmic reticulum ATPase.
Arch. Biochem. Biophys., 138: 285-294.

Janis, R.A., Crankshaw, D.J. and Daniel, E.E. (1977) Control of intracellular Ca^{2+}-activity in rat myometrium.
Am. J.Physiol., 232: c50-c58.

Jilka, R.L., Martonosi, A.N., Tillack, T.W. (1975) Effect of the purified (Mg^{2+} + Ca^{2+})- activated ATPase of sarcoplasmic reticulum upon the passive Ca^{2+} permeability and ultrastructure of phospholipid vesicles.
J.Biol. Chem., 250: 7511-7524.

Kanazawa, T., Yamada, S., Yamamoto, T. and Tonomura, Y. (1971) Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skelital muscle.
J.Biochem., 70: 95-123.

Knowles, A.F., Eytan, E., and Racker, E. (1976) Phospholipid-protein interactions in the Ca^{2+}-adenosine triphosphatase of sercoplasmic reticulum.
J.Biol. Chem., 251, 5161-5165.

Langer, G.A. (1968) Ion fluxes in cardiac excitation and contraction and their relation to myocardial contractivity.
Physiol. Rev., 48: 708-757.

Lee, K.S. and Shin, B.C. (1969) Studies on the active transport of calcium in human red cells.
J.Gen. Physiol., 54: 713-729.

LeMaire, M., Jørgensen, K.J., Røigaard, H. and Møller, J.V. (1976) Properties of deoxycholate solubilized sarcoplasmic reticulum Ca^{2+}-ATPase.
Biochemistry, 15: 5805-5812.

LeMaire, M., Møller, J.V. and Thanford, C. (1976) Retention of enzyme activity by detergent solubilized sarcoplasmic Ca^{2+}-ATPase.
Biochemistry, 15: 2336-2342.

Louis, C. and Shooter, E.M. (1972) The protein of rabbit skeletal muscle sarcoplasmic reticulum.
Arch. Biochem. Biophys., 153: 641-655.

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.

Luthre, M.G., Hildenbrandt, G.R. and Hanahan, D.J. (1976) Studies on an activator of the (Ca^{2+} + Mg^{2+})-ATPase of human erythrocyte membranes.
Biochim. Biophys. Acta 419, 164-179.

Ma, S.W.Y., Shami, Y., Messer, H.H. and Copp, D.H. (1974) Properties of Ca^{2+}-ATPase from the gill of rainbow trout (Salmo gardeneri).
Biochim. Biophys. Acta, 345: 243-251.

McFarland, B.H. and Inesi, G. (1971) [illegible] of solubilized sarcoplasmic reticulum.
Biochem. Biophys. Res. Commun., [illegible]

McFarland, B.H. and Inesi, G. (1971) [illegible] of sarcoplasmic reticulum [illegible]
Arch. Biochem. Biophys., [illegible]

McIntyre, J.D. and Green, J., (1974) [illegible] calcium transport in [illegible] human erythrocyte membranes by [illegible] cytoplasmic activator.
Biochim. Biophys. Acta, [illegible]

McIennan, D.H. (1970) [illegible] of an adenosine triphosphatase from [illegible] reticulum.
J.Biol. Chem., [illegible]

[illegible], D.S., [illegible], F.L. and [illegible], [illegible] properties of the [illegible] stimulated adenosine triphosphatase of [illegible] skeletal muscle.
Biochem. J., [illegible]

[illegible], A. (1966) The permeability of the [illegible] during active calcium [illegible]
Exp. Cell Research, [illegible]

[illegible], R. and Lee, A. (1967) [illegible] and calcium-dependent adenosine triphosphatase from the [illegible]
Biochem. Soc. [illegible]

[illegible], [illegible] and [illegible], in [illegible] (1973) [illegible] of calcium [illegible] sarcoplasmic reticulum [illegible]
[illegible]

[illegible], [illegible] and [illegible], (1973) [illegible] - stimulated calcium [illegible] [illegible]
[illegible]
[illegible]

Martonosi, A. (1968) Sarcoplasmic reticulum IV Solubilization of microsomal adenosine triphosphatase.
J.Biol. Chem., 243: 71-81.

Martonosi, A. (1969) Sarcoplasmic reticulum VII Properties of a phosphoprotein intermediate implicated in calcium transport.
J.Biol. Chem., 244: 613-620.

Martonosi, A. (1972) in Current Topics in Membrane and Transport (Bronner, F., Kleinzeller, A., eds) Vol. 3, pp 83-197, Academic Press, New York.

Martonosi, A., Donley, J. and Halpin, R.A. (1968) Sarcoplasmic reticulum III The role of phospholipids in the adenosine triphosphatase activity and Ca^{2+}-transport.
J.Biol. Chem., 243: 61-70.

Martonosi, A. and Feretos, R. (1964) Sarcoplasmic reticulum II Correlation between adenosine triphosphatase activity and Ca^{2+} uptake.
J.Biol. Chem., 239: 659-668.

Martonosi, A., Nakamaru, H., Jilka, R.L. and Vanderkooi, J.E. (1977) in Proceedings in the Life Sciences, FEBS Symp. No. 42 (Semenza, G. and Carafoli, E. eds.) pp 401-415 (Springer-Verlag, Berlin).

Meissner, G. (1973) ATP and Ca^{2+} binding by the Ca^{2+} pump protein of sarcoplasmic reticulum.
Biochim.Biophys. Acta, 298, 906-928.

Meissner, G., Conner, G.E. and Fleischer, S. (1973) Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+} pump and Ca^{2+} binding proteins.
Biochim. Biophys. Acta, 298: 246-269.

Meissner, G. and Fleischer, S. (1971) Characterization of sarcoplasmic reticulum from skeletal muscle.
Biochim. Biophys. Acta, 241: 356-378.

Melancon, M.J. and DeLuca, H.F. (1970) Vitamin D stimulation of calcium dependent adenosine triphosphatase in chick intestinal brush border. Biochemistry, 9: 1658-1664.

Mohri, H., Mohri, T. and Ernster, T. (1965) Isolation and enzymic properties of the mid piece of bull spermatozoa.
Expt. Cell.Res., 38: 217-246.

Moore, C. (1971) Specific inhibition of mitochondrial Ca^{2+}-transport by ruthenium red.
Biochem. Biophys. Res. Commun., 42: 298-305.

Moreland, R.S. and Ford, G.D. (1981) The influence of magnesium on calcium-activated vascular smooth muscle actomyosin ATPase activity.
Arch. Biochem. Biophys., 208: 325-333.

Morita, Z.and Chang, M.C. (1970) The motility and aerobic metabolites of spermatozoa in laboratory animals with special reference to the effects of cold shock and the importance of calcium for the motility of hamster spermatozoa.
Biol. Reprod., 3: 169-179.

Morton, B., Harrigen-Lum, J., Albagli, L. and Jooss, T. (1974) The activation of motility in quiscent hamster sperm from the epididymis by calcium and cyclic nucleotides.
Biochem. Biophys. Res. Commun., 56: 372-379.

Nakamaru, Y., Kosakai, M. and Konishi, K. (1967) Some properties of brain microsome adenosine triphosphatases activated by magnesium and calcium.
Arch. Biochem. Biophys., 120: 15-21.

Nakao, T., Nagano, Adachi, K. and Nakao, M. (1963) Separation of two adenosine triphosphatase from erythrocyte membrane.
Biochim. Biophys. Res. Commun. 13: 444-448.

Needham, D.M. and Williams, J.M. (1963) Proteins of the uterine contractile mechanism.
Biochem. J., 89: 552-561.

Ohnishi, T. and Ebashi, S. (1964) The velocity of calcium binding of isolated sarcoplasmic reticulum.
J.Biochem., 55: 599-603.

Olson, E.J. and Cazort, R.J. (1969) Active calcium and strontium transport in human erythrocyte ghosts.
J.Gen. Physiol., 53: 311-321.

Packer, L., Mehard, C.W., Meissner, G., Zahler, W.L. and Fleischer, S. (1974) The structural role of lipids in mitochondrial and sarcoplasmic reticulum membranes.
Biochim. Biophys. Acta, 363: 159-181.

Panet, R., Pick, U. and Selinger, Z. (1971) The role of calcium and magnesium in the adenosine triphosphatase reaction of sarcoplasmic reticulum.
J.Biol. Chem., 246: 7349-7356.

Parkinson, D.K. and Radde, I.C. (1971) Properties of a Ca^{2+} and Mg^{2+}-activated ATP-hydrolyzing enzyme in rat kidney cortex.
Biochim. Biophys. Acta, 242: 238-246.

Pagell, A. and Martonosi, [illegible] (1971) Sarcoplasmic reticulum. XIV Acetyl phosphate and carbamyl phosphate as energy sources for Ca^{2+}-transport.
J.Biol. Chem., 246: 5389-5397.

Quist, E.E. and Roufogalis, B.D. (1975) Calcium transport in human erythrocytes. Separation and reconstitution of high and low affinity $(Mg^{2+}-Ca^{2+})$-ATPase activities in membranes prepared at low ionic strength.
Arch. Biochim. Biophys., 166: 240-251.

[illegible], E. (1970) Cell communication, calcium ion and cyclic adenosine monophosphate.
Science, 170: 404-412.

Rooblee, L.S., Shepro and Belamarich, F.A. (1973) Calcium uptake and associated adenosine triphosphatase activity of isolated platelet membranes. J.Gen. Physiol., 61: 462-481.

Rosenthal, A.S., Kregenow, F.M. and Moses, H.L. (1970) Characteristics of a Ca^{2+}-dependent ATPase activity associated with a group of erythrocyte membrane proteins which form fibrils. Biochim. Biophys. Acta, 196: 254-262.

Rubin, B.B. and Katz, A.M. (1967) Sodium and potassium effects on skeletal muscle microsomal adenosine triphosphatase and calcium uptake. Science, N.Y. 158: 1189-1191.

Scharff, O. and Foder, B. (1978) Reversible shift between two states of Ca^{2+}-ATPase in human erythrocytes mediated by Ca^{2+} and a membrane bound activator. Biochim. Biophys. Acta, 509: 67-77.

Schatzmann, H.J. (1967) Ca-activated membrane ATPase in human red cells and its possible role in active Ca-transport. Protides of the biological fluids, Vol. 15, pp. 251-255. Amsterdam: Elsevier.

Schatzmann, H.J. (1973) Dependence on calcium concentration and stoichiometry of the calcium pump in human red cells. J.Physiol. (London), 235: 551-569.

Schatzmann, H.J. (1975) Active calcium transport and Ca^{2+}-activated ATPase in human red cells. Curr. Top. Membr. Transp., 6: 125-166.

Schatzmann, H.J. and Vincenzi, F.F. (1971) $(Ca^{2+}-Mg^{2+})$-activated membrane ATPases in human red cells and their possible relation to cation transport. Biochem. Biophys. Acta, 241: 379-392.

Schatzmann, H.J. and Vincenzi, F.F. (1969) Calcium movements across the membrane of human red cells. J.Physiol. (London), 201: 369-395.

Shami, Y. and Radde, I.C. (1971) Calcium stimulated ATPase of guinea pig placenta. Biochim. Biophys. Acta, 249: 345-352.

Singh, J.P. (1980) Calcium in acrosome reaction and flagellar contractility of mammalian spermatozoa. Ph.D. Thesis, Univ. Wisconsin-Madison, USA.

Singh, J.P., Babcock, D.F. and Lardy, H.A. (1978) Increased calcium-ion influx is a component of capacitation of spermatozoa. Biochem. J., 172: 549-556.

Skou, J.C. (1965) Enzymic basis for active transport of Na^+ and K^+ across cell membrane. Physiol. Rev., 45: 596-617.

Sonnenblick, E.H. and Stam, A.C. Jr. (1969) Cardiac muscle: activation and contraction. Ann. Rev. Physiol., 31: 647-674.

Suko, J. and Hasselbach, W. (1976) Characterization of cardiac sarcoplasmic reticulum ATP-ADP phosphate exchange and phosphorylation of the calcium transport adenosine triphosphatase. Eur. J. Biochem., 64: 123-130.

Taylor, C.R. (1962) Cation stimulation of an ATPase system from the intestinal mucosa of the guinea pig. Biochim. Biophys. Acta, 60: 437-440.

The, R. and Hasselbach, W. (1972) Properties of the sarcoplasmic ATPase reconstituted by oleate and lysolecithin after lipid depletion. Eur. J. Biochem., 28: 357-363.

Thorens, S. (1979) Ca^{2+}-ATPase and Ca^{2+}-uptake without requirement for Mg^{2+} in membrane fractions of vascular smooth muscle. FEBS Lett., 98: 177-180.

Verity, A.A. and Bevan, J.A. (1969) Membrane adenosinetriphosphatase activity of vascular smooth muscle.
Biochem. Pharmacol., 18: 327-338.

Vijayasarathy, S., Shivaji, S. and Balaram, P. (1980) Plasma membrane bound Ca^{2+}-ATPase activity in bull sperm.
FEBS Lett., 114: 45-47.

Vincenzi, F.F. (1968) The calcium pump of erythrocyte membrane and its inhibition by ethacynic acid.
Proc. Western Pharmacol. Soc., 11: 58-60.

Walton, A. (1945) Notes on artificial insemination of sheep, cattle and horse. Holborn Surgical Instruments Co., London.

Wathanabe, T. and Flavin, M. (1973) Two types of adenosine triphosphatase from flagella of Chlamydomonas reinhardi.
Biochim. Biophys. Res. Commun., 52: 195-201.

Watson, E.L., Izutsu, K.T. and Siegel, I.A. (1974) Calcium stimulated ATPase of dog submandibular gland.
Arch. Oral. Biol. 19: 13-16.

Weber, A. (1966) The role of Ca in the regulation of muscle activity. Myocardial cell, struct., funct., modif. cardiac drugs, heart ass. southeast pa., Int. Symp. 131-143.

Weber, A., Herz, R. and Reiss, I. (1966) Kinetics of Ca transport by isolated fragmented sacroplasmic reticulum.
Biochem. Z., 345: 329-369.

Weber, A. and Sanadi, R. Current topics in bioenergetics, Vol. 1 Academic Press, New York, 1966, p 203.

Weidekamm, E., and Bridczka, D. (1975) Extraction and localization of a (Ca^{2+} + Mg^{2+})- stimulated ATPase in human erythrocyte spectrin. Biochim. Biophys. Acta, 401: 51-58.

Weiner, M.L. and Lee, K.S. (1972) Active calcium ion uptake by inside-out and right side-out vesicles of red blood cell membranes. J.Gen. Physiol., 59: 462-475.

Wins, P. and Schoffeniels (1966 a) Studies on red cell ghost ATPase system. Properties of a (Mg^{2+} + Ca^{2+}) - dependent ATPase. Biochim. Biophys. Acta, 120: 341-350.

Wolf, H.U. (1970) Purification of the Ca^{2+}-dependent ATPase of human erythrocyte membranes. Biochem. Biophys. Acta, 219: 521-524.

Wolf, H.U. (1972) Studies on a Ca^{2+}-dependent ATPase of human erythrocyte membranes. Effects of Ca^{2+} and H^{+}. Biochem. Biophys. Acta, 266: 361-375.

Wooding, F.B.P. (1973) The effect of Triton X-100 on the ultrastructure of ejaculated bovine sperm. J.Ultrast. Res., 42: 502-516.

Wooding, F.B.P. and O'Donnell (1971) A detailed ultrastructural study of the head membranes of ejaculated bovine sperm. J.Ultrastruct. Res., 35: 71-85.

Wuytack, F. and Casteels, R. (1980) Demonstration of a (Ca^{2+} + Mg^{2+}) - ATPase activity probably related to Ca^{2+}-transport in the microsomal fraction of porcine coronary artery smooth muscle. Biochim. Biophys. Acta, 595: 257-263.

Wuytack, F., Schutter, G.D. and Casteels, R. (1981) Partial purification of (Ca^{2+} + Mg^{2+}) - dependent ATPase from pig smooth muscle and reconstitution of an ATP-dependent Ca^{2+}-transport system. Biochem. J., 198: 265-271.

Yamada, S. and Tonomura, Y. (1972) Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle VII Recognition and release of Ca^{2+} ions.
J.Biochem., 72: 417-425.

Yamada, S., Yamamoto, T. and Tonomura, Y. (1970) Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. III Ca^{2+}-uptake and ATP splitting.
J.Biochem., 67: 789-794.

Yamamoto, T. and Tonomura, Y. (1967) Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle I Kinetic studies.
J.Biochem., 62: 558-575.

Yamamoto, T. and Tonomura, Y. (1968) Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. II Intermediate formation of phosphoryl protein.
J.Biochem., 64: 137-145.

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