CONFORMATIONAL CHANGES IN THE (Ca^{2+},Mg^{2+})-ATPase OF SARCOPLASMIC RETICULUM DURING ENERGY TRANSDUCTION

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AMPPCP	β, γ -methylene adenosine 5' -triphosphate
AMPPNP	Adenosine 5' - (β, γ-imino) triphosphate
CDTA	Trans- 1, 2-diaminocyclohexonetetra-acetic acid
DOC	Deoxycholate
DTNB	5,5' -dithiobis (2- nitrobenzoate)
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol bis (β-aminoethyl ether) N,N' -tetraacetic acid
EP	Phosphoenzyme
LDH	Lactate dehydrogenase
NADH	Nicotinamide-adenine dinucleotide
NEM	N-ethylmaleimide
PEP	Phosphoenolpyruvate
PK	Pyruvate kinase
Pi	Inorganic phosphate
SDS	Sodium dodecyl sulphate
-SH	Sulphydryl
SR	Sarcoplasmic reticulum
SRV	Sarcoplasmic reticulum vesicles
-S-S-	Disulphide
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine

ABSTRACT

Treatment of SR membranes with mild acid (pH 5.6) (Berman, M.C., McIntosh, D.B. and Kench, J.E. (1977) J. Biol. Chem. 252, 994-1001) or incubation with millimolar concentrations of ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) at neutral pH and 37°C (McIntosh, D.B. and Berman, M.C. (1978) J. Biol. Chem. 253, 5140-5146) results in a progressive irreversible inhibition of calcium transport while (Ca^{2+}, Mg^{2+})- ATPase activity is unimpaired. Possible conformational changes associated with this uncoupling were monitored by following alterations in kinetic mobility of sulphydryl (-SH) groups either by using 5, 5'-dithiobis- (2-nitrobenzoate) (DTNB) and stopped flow analysis or 1-^{14}C-N-ethylmaleimide (NEM). Kinetic reactivity with DTNB revealed a total of 20 thiol groups/1.5×10^5 g of SR protein (containing 1 mole of ATPase protein) in the presence of sodium dodecyl sulphate, which constitute four kinetic classes. In native control vesicles 4.5 thiol groups were unreactive, 0.4 represented the fast reacting class, 0.8 the moderately fast reacting class and 14.4 the slowly reacting class, displaying pseudo-first order rate constants, k, of 159.0-, 22.0- and 0.23×10^{-2} sec^{-1}, respectively. Inactivation of calcium transport to the extent of 90%, using mild acid conditions, increased the number of fast and moderately fast reacting groups, each by 1.0 - 1.5 sulphydryl groups/mol ATPase. The number of slowly reacting groups decreased by

approximately 3.0 thiol groups/mol ATPase. The kinetics of the reaction with 1-^{14}C-NEM was essentially similar to that with DTNB. EGTA inactivation of calcium transport, to the extent of 90% and subsequent 1-^{14}C-NEM modification, resulted in an increase in the number of fast reacting thiol groups by 0.5-1.0 thiol groups/mol ATPase. The total number of reactive thiol groups decreased by 1.0 - 2.0 thiol groups/mol ATPase, probably due to autoxidation of the newly exposed sulphydryl group.

Inactivation of transport carried out in the presence of N-ethylmaleimide to prevent autoxidation resulted in an increase of approximately one thiol group/mol ATPase. The rate constant for the increase in reactivity of this group was 1.45 min^{-1}. This thiol group was localized on the ATPase protein of molecular weight approximately 100 000 daltons. Trypsinization of the ATPase produced four fragments of molecular weights 55 000, 45 000, 30 000 and 20 000. More extensive cleavage resulted in a significant decrease in the 55 000 dalton fragment and increased amounts of the 30 000 and 20 000 dalton subfragments. There was increased labelling on all subfragments of EGTA-treated vesicles compared to control, untreated vesicles. However, the greatest relative increase in labelling appeared to be localized on the 55 000 dalton and 20 000 dalton subfragments. Peptide mapping of the purified ATPase revealed 24 ninhydrin-positive peptides. Five of these were labelled in control and EGTA-treated vesicles, four of which showed increased labelling

in the latter preparation. Random labelling of the non-overlapping fragments may be due to the enzyme being "trapped" in a number of intermediate conformations or due to heterogeneity within the ATPase populations.

NEM modification of SR membranes did not affect the tryptic cleavage pattern or the mobilities of the tryptic subfragments. It did however, affect the extent of tryptic cleavage resulting in solubilization of NEM-labelled protein into the medium following centrifugation. This protein fraction was identified as consisting largely of the 55 000 dalton molecular weight species on sodium dodecyl sulphate gel electrophoresis.

It is concluded that occupancy of high affinity ($K_{0.5(Ca^{2+})} \approx 10^{-6}M$) calcium binding sites maintain the (Ca^{2+}, Mg^{2+})- ATPase in a stable, coupled conformation. Displacement of this calcium induces a conformational change in the protein which results in the loss of the vectorial component of calcium transport.

1.0 INTRODUCTION

1.1 BRIEF OVERVIEW AND AIM OF STUDY

Isolated vesicles of sarcoplasmic reticulum (SR) prepared from rabbit skeletal muscle translocate calcium by a process which is coupled to ATP hydrolysis. The molecular species identified with this process is the (Ca^{2+}, Mg^{2+}) -ATPase (EC 3.6.1.3), with a molecular weight approximately 100 000 (MacLennan, 1970) and which constitues 70-80% of the membrane proteins. The catalytic cycle has been separated into a number of partial reactions with at least eight intermediates (Verjovski-Almeida et al., 1978). Several of these intermediates have been characterized by sulphydryl group reactivity (Murphy, 1976 and 1978; Yamada and Ikemoto, 1978), sulphydryl group-directed spin labels (Champeil et al., 1976 and 1978; Coan et al. 1977 and 1979), sulphyrdryl group-directed fluorescent probes (Ikemoto et al., 1978), tryptophan fluorescence (Dupont, 1976 and 1977), ligand-induced stabilization (McIntosh and Berman, 1978) and kinetic behaviour (de Meis and Carvalho, 1974) and shown to be different conformational states of the ATPase. Energy for transport appears to be provided in part from ligand binding energy and in part from the hydrolysis of ATP. The transfer of calcium ions from the outside to the inside of the SR vesicles follows extremely rapidly on phosphorylation of the enzyme (Kurzmack et al., 1977) and some evidence suggests that it is accompanied by a transposition of high affinity sites on the other side (Ikemoto, 1975). Normally ATP hydrolysis is tightly coupled to calcium transport in a ratio of 1:2, respectively

(Hasselbach and Makinose, 1963; Weber et al., 1966). However, at low pH (Berman et al.,1977) and low substrate concentrations (Rossi et al., 1979) ATP hydrolysis does not support calcium transport. An "apparent uncoupling" also accompanies treatment of the vesicles by such agents as detergents (McFarland and Inesi, 1970 and 1971), organic solvents (Inesi et al., 1967), and phospholipases (Martonosi and Fortier, 1974; Fiehn and Hasselbach, 1970). However, in these instances the inhibition of the calcium transport is considered to be due to enhanced "leakiness" of the vesicles to calcium through their action on the liquid components of the membrane. Prolonged incubation of SR vesicles under alkaline conditions in the presence of EGTA has also been used to prepare "leaky" vesicles (de Meis, 1976). This treatment enhances the accessibility of the vesicle lumen to inulin and releases lumenally located extrinsic proteins (Duggan and Martonosi,1970). Hence it appears that the membrane structure is disrupted without apparently affecting the ATPase. However, under milder conditions, at neutral pH, which does not render the vesicles permeable to inulin nor result in loss of protein (McIntosh and Berman, 1978), calcium transport is also abolished. Mild acid conditions have a similar effect (Berman et al., 1977). The characteristics of the inactivation, including stabilization of transport via high affinity calcium binding sites and large entropic and enthalpic contributions, suggest a partial denaturation or minor perturbation of the ATPase.

Uncoupling of transport by mild acid treatment or by EGTA at neutral pH is irreversible (Berman et al., 1977; McIntosh and Berman, 1978). The aim of this study was to attempt to characterize the irreversible conformational change in the ATPase accompanying uncoupling of calcium transport by these procedures. The parameter chosen to monitor changes in protein conformation was that of sulphydryl group kinetic reactivity, since kinetic reactivities and accessibility of -SH groups in proteins has previously been shown to be extremely sensitive to minor perturbations in protein conformation in a wide variety of protein species (Boyer, 1959; Friedman, 1973; Torchinskii, 1974; Jocelyn, 1972).

1.2 STRUCTURE AND COMPOSITION OF ISOLATED SARCOPLASMIC RETICULUM MEMBRANE

The membrane system of the isolated sarcoplasmic reticulum, consisting almost exclusively of the ATPase protein (one polypeptide), provides one of the simplest and most direct models of attempting to elucidate the molecular mode of energy transduction.

The membrane system in muscle cells in vivo, which has a role in controlling muscle contraction, consists of two main components (a) the plasma membrane with its tubular unfoldings (the T-system) running transverse to the fibre axis and (b) a highly differentiated endoplasmic reticulum, the sarcoplasmic reticulum (SR). The sarcoplasmic reticulum forms a membranous network, consisting of continuous tubules and cisternae, surrounding the myofibrils and forming separate enclosed

compartments within muscle cells (Bennett and Porter, 1953; Porter and Palade, 1957; Franzini-Armstrong et al., 1975). The T-system forms a junction with the terminal cisterna, a part of the sarcoplasmic reticulum that is thickened to form a continuous sac. The membranes of the T-system are continuous with the surface plasma membrane, the sarcolemma, and thus the lumen is a continuation of the extracellular fluid compartment (Endo, 1964; Franzini-Armstrong et al., 1975; Huxley, 1964). On both sides of each T-tubule, at the level of the A-I junction of sarcomeres, two terminal cisternae are situated in close association with the T-tubules forming a triad. There is no direct communication between different parts of the triad. The T-tubules and terminal cisternae are separated by a gap of 120-140 $\mathring{A}$ (Franzini-Armstrong, 1970).

Muscle cells possess both a calcium-sensitive contractile system and a calcium-accumulating membrane system (Bozler, 1954; Watanabe, 1955; Weber and Herz, 1961). It was demonstrated by Hasselbach and Makinose (1961) and Ebashi and Lipman (1961, 1962), that muscle microsomes (SR) were capable of accumulating Ca^{2+} in the presence of ATP and Mg^{2+} by means of an ATPase "pump" protein. Thus it was postulated that calcium released from the sarcoplasmic reticulum can induce muscle contraction, whilst relaxation is brought about by calcium accumulation by the sarcoplasmic reticulum.

Isolation of Sarcoplasmic Reticulum Membranes

Sarcoplasmic reticulum vesicles are prepared by vigorous homogenization of rabbit skeletal muscle, which causes the membranes to fragment, but they reseal to form vesicles which retain the proper orientation for Ca^{2+} transport (Ebashi and Lipman, 1962; Nagai et al., 1960). Microsomal fractions of these vesicles are isolated by differential centrifugation of the muscle homogenates in isotonic salt solution (Portzehl, 1957) in the presence of sucrose (McFarland and Inesi, 1971; Ikemoto et al., 1971). Contaminating actomyosin is removed by solubilization in 0.6 M KCl, which does not disrupt the sarcoplasmic reticulum protein (Martonosi, 1968). These preparations are relatively pure and have a high enzymatic activity.

Composition of the SR Membranes

Protein Composition

SR membranes contain four major proteins, all of which are considered to be related, to some extent, to the function of calcium transport. Martonosi and Halpin (1969a, 1971) separated 3 of these major proteins of the SR by SDS-polyacrylamide gel electrophoresis. These three proteins are the ATPase protein (MW ≈ 100 000) (MacLennan, 1970; Inesi, 1972), a high affinity calcium-binding protein (MW ≈ 56 000) and calsequestrin (MW ≈ 44 000) (MacLennan and Wong, 1971; Ostwald and MacLennan, 1974). There are additional minor proteins in

the SR membranes, but their properties are not well defined. Estimation of the protein composition of the membranes by measurement of the density profile of stain proteins after disc-gel electrophesis, indicates that the ATPase accounts for 60-80% of the total protein, the high-affinity calcium binding protein accounts 5-12% and the calsequestrin for 5-19% (Inesi, 1972; Malan et al., 1975; Ikemoto et al., 1971; Meissner et al., 1973). However, Meissner (1975) found two populations of SR vesicles by separation on a sucrose-density gradient, each having different protein compositions. The light vesicles are comprised mostly of ATPase (approx. 90%), calsequestrin and the calcium-binding protein, are virtually absent, whereas heavy vesicles contain the ATPase (55-60%), calsequestrin (20-25%), and the high-affinity calcium-binding protein (5-7%). The proteins of the sarcoplasmic reticulum can be classified as either extrinsic or intrinsic membrane proteins. The calcium-binding protein and calsequestrin constitute the extrinsic group which are loosely associated membrane proteins and are easily removed by EGTA or EDTA washes at slightly alkaline pH (Duggan and Martonosi,1970; Meissner et al., 1973). The (Ca^{2+},Mg^{2+}) -ATPase constitutes the intrinsic group of proteins which together with the phospholipid form the primary structure of the membrane. These proteins require rather harsh physical conditions for solubilization (Meissner et al., 1973).

The (Ca^{2+}, Mg^{2+}) -ATPase

The Ca^{2+}-pump protein, a single species of polypeptide of approx, 100 000 daltons, was deduced to be the (Ca^{2+}, Mg^{2+}) -ATPase by Martonosi and Halpin (1971), since ^{32}P from [γ-^{32}P] -ATP is exclusively incorporated into this component. MacLennan (1970) purified the ATPase protein, with a two-fold increase in its activity, by treating the sarcoplasmic reticulum membrance with low concentrations of deoxycholate in the presence of high ionic strength (1M KCl). This procedure, which extracted the extrinsic and contaminating proteins, was followed by dissolving the ATPase protein remaining in the membrane with higher concentration of deoxycholate and fractionating the solution with ammonium acetate. The purified ATPase catalyses a (Ca^{2+}, Mg^{2+})-dependent ATP hydrolysis (MacLennan et al., 1971) and ATP − ADP exchange reaction. The purified ATPase contains about 530 µg phospholipid/mg protein, an amount almost equal to that found in intact SR (MacLennan, 1970; MacLennan et al., 1971). The ATPase protein was also purified by Ikemoto et al. (1971); MacFarland and Inesi (1971); Meissner et al. (1973); Le Maire et al. (1976); Martonosi (1968); Deamer (1973) and Warren et al. (1974a) using detergents, such as Triton X-100, deoxycholate, lysolecithin and dodecyloctaoxyethylene glycol monoether, to solubilize the membrane.

Calsequestrin

Calsequestrin was first isolated and characterised by MacLennan and Wong (1971). They postulated that the protein is a major site of Ca^{2+} sequestration in the interior of sarcoplasmic reticulum membrane vesicles or sacs. Calsequestrin is a protein of strong negative charge, with a molecular weight of approx. 44 000 (MacLennan and Wong, 1971). However, the values for the molecular weight vary between 44 000 and 65 000 daltons (Ikemoto et al., 1971, 1972; MacLennan, 1974; Meissner et al., 1973; Ostwald et al, 1974). The apparent molecular weight of calsequestrin changed following minor changes in the condition of electrophoresis. Calsequestrin is an acidic protein, being suitable for sequestering calcium, and about 37% of the total amino acid residues are comprised of equal amounts of glutamic and aspartic acid residues and less than 9% of the residues are basic. In the presence of 100 mM KCl, calsequestrin has a high capacity for binding Ca^{2+} (850 nmoles Ca^{2+}/mg) and low affinity. The dissociation constant for Ca^{2+} is approximately 800 μM (Ostwald and MacLennan, 1974). Calsequestrin would not be expected to bind a sufficient amount of calcium in the presence of sarcoplasmic Ca^{2+} concentrations of less than 1 μM (Weber, 1966). However, the interior of the sarcoplasmic reticulum may contain Ca^{2+} concentrations of 10-20 mM (if it were free) (MacLennan and Wong, 1971; Sandow, 1970). This intravesicular calcium concentration in the ionized form, would be sufficient to saturate the calcium binding sites of calsequestrin, if the calsequestrin was situated on interior sites. Thus it has

been suggested that calsequestrin may play a role in binding calcium ions which have been actively translocated into the lumen of the vesicle (MacLennan and Wong, 1971; Jilka et al., 1975; Garcia et al., 1975), rather than participating as an integral part of the calcium pump. There is doubt as to the location of calsequestrin in the interior, or exterior surface of the membrane. However, direct evidence from the extraction data of MacLennan and Wong (1971), and studies with antibodies raised against calsequestrin which fail to aggregate sarcoplasmic reticular membranes (Stewart et al., 1976), indicate that the protein is not on the external surface. Stewart and MacLennan (1974) also reported that calsequestrin, in intact vesicles, is relatively resistant to proteolytic cleavage by trypsin. An internal location has been suggested by Duggan and Martonosi (1970) who provided evidence for the extraction of calsequestrin with EDTA. However, this extraction with EDTA made the membranes permeable to inulin and thus the localization could not be determined. Lacterperoxidase labelling of sarcoplasmic reticulum did not reveal the location of calsequestrin since all the proteins in the membrane were labelled with ^{125}I in the presence of the enzyme (MacLennan et al., 1972 ; Thorley-Lawson and Green, 1973). In summary, from the weight of the evidence, an internal localization of calsequestrin is favoured.

High Affinity Calcium Binding Protein

The high affinity calcium binding protein or M_{55} protein has a molecular weight of 55 000 and was first isolated by Ostwald and MacLennan (1974). This protein is also acidic, but not as acidic as calsequestrin. It binds Ca^{2+} with a high affinity, but low capacity (16-22 nmoles/mg) (Ikemoto et al., 1974; Ostwald and MacLennan, 1974). In the presence of 100mM KCl, Ostwald and MacLennan (1974) found that the dissociation constant was between 2.5 to 4 μM. The M_{55} protein and calsequestrin appear to have a similar internal location, as shown by extraction with EDTA (Duggan and Martonosi, 1970), lactoperoxidase-catalysed-iodination (MacLennan et al., 1972; Thorley-Lawson and Green, 1973), antibody tagging and trypsin digestion (Stewart et al., 1974, 1976).

Proteolipid

MacLennan et al. (1972) reported that the proteolipid constitutes another intrinsic protein of the sarcoplasmic reticulum. It was isolated by MacLennan et al. (1972) by sequential extraction with organic solvents. This protein accounted for only a few percent of the total staining material in SDS gels, and was identified as a band with a molecular weight of about 6 000. Amino acid analysis, however, indicated a molecular weight of 12 000. Racker and Eytan (1975) found that a heat stable factor, resembling the proteolipid,

increased the coupling ratio for transport in reconstitution experiments with the purified ATPase and exogenous phospholipid vesicles. However, the function and physiological role of the proteolipid is still to be determined.

Lipid Composition of SR

In SR molecules, the amphiphillic (Ca^{2+}, Mg^{2+}) -ATPase is surrounded by lipids, consisting of approximately 80% phospholipids and 20% neutral lipid. This total lipid accounts for 40% of the dry weight of the membrane. The phospholipids of SR consist of phosphatidylcholine (65-73%), phosphatidylethanolamine (12-19%), phosphatidylinositol (±9%), phosphatidylserine (±2%), sphingomyelin (±4%) and cardiolysin (0.1-0.3%) (Martonosi et al., 1968; Meissner and Fleischer, 1971; Owens et al., 1972; Waku et al., 1971). The neutral lipid consists mainly of cholesterol, but contains small amounts of triglyceride and free fatty acids (Marai and Kuksis, 1973). The role of the individual lipids in calcium transport and ATPase activity is not clearly understood. The reconstitution experiments of Knowles et al. (1976) show that although phosphatidylcholine alone is effective in restoring the Ca^{2+} -ATPase activity of the delipidated enzyme, this component alone does not support ATP-dependent Ca^{2+} transport, whilst phosphatidylethanolamine, on its own, supports both ATPase and calcium transport activity. Approximately 30 out of about 90 lipid molecules, associated with each molecule of ATPase in native SR, interact directly with the ATPase (Hesketh et al., 1976; Warren et al., 1974a,

1974b, 1975), the remainder of the lipid molecules contribute to the fluid bilayer characteristics. Jost et al.(1973) suggested that a boundary between the "bulk" lipid of the membrane and the protein itself was provided by an annulus layer of phospholipids in direct contact with the hydrophobic region of the protein. This helped to maintain the protein in its proper conformation. A boundary lipid surrounding the protein in the membrane of sarcoplasmic reticulum was first reported by Warren and coworkers (1974a, b). They found 30 mol of lipid tightly bound per mol of ATPase constituting the annulus around the protein. Delipidation of the purified ATPase by deoxycholate treatment led to loss of ATPase activity when a ratio of 15 mol of bound lipid per ATPase was obtained. Chapman (1979), however, pointed out that proteins may trap the lipid between them and this minimum amount of lipid may be required merely to retain the appropriate fluidity for the protein and thus prevent protein aggregation which is irreversible. This concept is in contrast to that of a special lipid annulus which proposes that the minimum amount of lipid associated with the protein is required for enzymatic activity (Warren et al., 1974a, b).

Ultrastructure of the SR Membrane and of the ATPase

Sarcoplasmic reticulum vesicles, in isolation, have four major features that are detectable by different electron microscopic preparations. Staining of thin layer sections of SR vesicles with osmium and with lead and uranium salts indicate

them to be single, hollow vesicular membranous structures with a trilaminar boundary (Ebashi and Lipman, 1962; Hasselbach and Elfvin, 1967; Nagai et al., 1960). Density staining of the two leaflets and electron microscopic studies of the binding of Hg-phenylazoferritin to SR membranes indicate an asymmetry of protein with a greater concentration on the cytoplasmic surface (Hasselbach and Elfvin, 1967). X-ray diffraction studies of vesicles, packed by centrifugation, also indicated a protein asymmetry across the membrane (Dupont et al., 1973; Worthington and Liu, 1973). Dupont et al. (1973) found a greater percentage on the exterior surface, while Worthington and Liu (1973) indicated higher concentration of the protein on the inner surface. Problems regarding the definition of the internal and external surfaces of the membrane has led to controversy as to whether the pump protein is in the vesicular lumen or on the cytoplasmic surface. Negative staining provides a view of the surface of SR membranes. Ikemoto et al. (1968) and Inesi and Asai (1968) found, using negatively-stained electron-micrographs, that the surface of the SR membranes are covered with particles about 4 nm in diameter that are connected to the surface with stalks about 2 nm in diameter (Ikemoto et al., 1971). These projections are protein in nature since extensive tryptic digestion of the membrane led to a loss of these surface particles (Stewart and MacLennan, 1974; Inesi and Scales, 1974; Martonosi, 1968). Freeze-fracture of membranes, in which the leaflets of the bilayer may be separately visualized, reveals a single size of particle of about 8-9 nm embedded in the hydrophobic region

of the membrane (Deamer and Baskin, 1969; Baskin, 1971). The cytoplasmic leaflet contains a much greater proportion of particles than does the cisternal leaflet. The density of these particles is approximately $\frac{1}{3}$ - $\frac{1}{4}$ the density of negatively-stained particles on the vesicle surface. The freeze-fracture particles are randomly distributed with an average interparticle distance of 110 Å (Scales and Inesi, 1976). The negatively-stained projections on the outer surface are not destroyed by mild trypsin treatment, which cleaves the ATPase into subfragments (Scales and Inesi, 1976). However, more extensive trypsinization results in the disappearance of the outer projections (Ikemoto et al., 1968; Inesi and Asai, 1968) and this is correlated with a decrease in the number of particles in the outer bilayer leaflet accompanied by a simultaneous increase in the inner leaflet (Stewart and MacLennan, 1974; Inesi and Scales, 1974; Scales and Inesi, 1976). From these results it appears that the inner hydrophobic particle is confined to the outer leaflet of the bilayer by the outer hydrophilic projection , which has been shown to be part of the ATPase polypeptide as antibodies to the ATPase bound to it (Stewart et al., 1976).

An inconsistency between the densities of the surface projections and of the intramembranous particles was found by Scales and Inesi (1976) and Jilka et al. (1975) and was thought to be probably due partly to the formation of oligomers containing three or four hydrophobic ends that appear as single intramembranous particles. However, the hydrophilic, polar regions on the surface of the membrane remained distinct

(Scales and Inesi, 1976). This inconsistency could, however, be explained by variable penetration of the polypeptide chain of the ATPase into the membrane. This suggestion is supported firstly by the evidence that the fully active detergent-solubilized ATPase has a minimal molecular weight of approximately 400 000 corresponding to a trimer or tetramer (Le Maire et al., 1976) and secondly by the cross-linking experiments of Murphy (1976a), who observed a species of molecular weight 400 000 on SDS-polyacrylamide gels after cupric phenanthroline oxidation. In addition, Murphy (1976a) observed no change in the electrophoretic mobilities of the calcium binding protein and calsequestrin and no oligomeric forms corresponding to dimeric, trimeric or pentameric forms of the ATPase.

Proteolytic Fragmentation of the ATPase

An understanding of the submolecular structure of the ATPase protein and the intramolecular distribution of the major functional activities of the protein are necessary for an elucidation of the mechanism of calcium transport. The ATPase molecule (MW 100 000) has been shown to consist of a hydrophilic region exposed to the exterior of the membrane, while its hydrophobic region is submerged in the membrane, interacting directly with the boundary phospholipids (Stewart et al., 1976). Brief tryptic digestion in the presence of 1M sucrose results in the cleavage of the ATPase protein into 2 fragments with molecular weights of 52 000 - 60 000 and 45 000 - 55 000 (single cleavage) (Inesi and Scales, 1974;

Migala et al., 1973; Stewart and MacLennan, 1974; Stewart et al., 1976; Thorley-Lawson and Green, 1973) (Table 1), as determined by SDS-polyacrylamide gel electrophoresis. The ATPase activity and calcium transport activity are unimpaired following limited cleavage of the molecule which does not appear to alter membrane morphology (Stewart and MacLennan, 1974; Inesi and Scales, 1974).

TABLE 1 Molecular Weights of the (Ca^{2+}, Mg^{2+}) -ATPase and its Tryptic Subfragments (from Tada et al., 1978).

ATPase	Single Tryptic Cleavage		Double Tryptic Cleavage		Reference
115 000	60 000	55 000	33 000	24 000	Thorley-Lawson and Green (1973, 1975)
102 000	55 000	45 000	30 000	20 000	Stewart and MacLennan (1974) Stewart et al. (1976) MacLennan et al. (1971)
100 000	60 000	49 000	32 000	21 000	Yamamoto and Tonomura (1977)
106 000	57 000	46 000			Inesi and Scales (1974)

Further proteolysis yields fragments of 30 000 and 20 000 molecular weight derived from cleavage of the 55 000 molecular weight fragment (double cleavage) (Stewart et al., 1976; Thorley-Lawson and Green, 1975). This cleavage results in uncoupling of ATP hydrolytic activity from Ca^{2+} transport.

Ultimately, on prolonged trypsinization, the ATPase is decreased to fragments with molecular weights of 5 000 or less. Degradation to the low molecular weight fragments (MW < 5 000) is associated with loss of surface particles and loss of ATPase activity. The globular particles in the membrane interior are rearranged, but not lost (Stewart and MacLennan, 1974; Inesi and Scales, 1974) and a large amount of the fragmented ATPase still remains associated with the lipid (Stewart and MacLennan, 1974). The single cleavage is instantaneous in the presence of IM sucrose and ATP, however, the double cleavage is extremely slow and further cleavage to the low molecular weight fragments undetectable. Presumably the ATPase is stabilized by the ATP and sucrose in a configuration in which the secondary cleavage sites are not readily exposed to trypsin (Inesi and Scales, 1974). Proteolytic subfragmentation of the (Ca^{2+}, Mg^{2+}) -ATPase in this orderly fashion is a very effective way of localizing the major functional properties of domains of the ATPase molecule. The 55K and 30K molecular weight subfragments are phosphorylated by [γ-^{32}P]ATP (Thorley-Lawson and Green, 1973; Stewart *et al.*, 1976) and the thiol group, protected by ATP (Hasselbach and Seraydarian, 1966; Panet *et al.*, 1971; Panet and Selinger, 1970), is labelled with N-ethyl-[2-^{3}H] maleimide (Stewart *et al.*, 1976), indicating that these subfragments contain the active site of ATP hydrolysis. The 55K molecular weight fragment is also iodinated to a greater extent in the presence of lactoperoxidase than the 45K molecular weight fragment (Thorley-Lawson and Green, 1973).

Stewart and MacLennan (1974) and MacLennan (1975) have isolated and analysed the amino acid composition of the 55K and 45K sugfragments. The 45K molecular weight fragment is more hydrophobic than the 55K fragment, containing 40% polar amino acids and 60% nonpolar amino acids. The larger fragment has equal amounts of polar and non-polar amino acids. Antibodies raised against the two fragments do not cross-react, indicating that the two fragments do not contain significant overlapping regions. The use of antibodies indicates that the 55K fragment is exposed to the surface of the membrane, while the smaller fragment is inaccessible (MacLennan, 1975; Stewart et al., 1976). These data suggest that the active site is located on the 55K fragment that is exposed to the external surface of the SR membrane, while the 45K fragment is embedded in the membrane. MacLennan et al. (1976) showed that the 55K and 45K fragments dissociated by brief exposure to solutions containing SDS, can be reconstituted into phospholipid vesicles (Racker et al., 1975) and are then capable of carrying out active calcium transport.

The 30K molecular weight fragment contains the hydrolytic site and the 20K fragment the ionophonic site (Thorley-Lawson and Green, 1973, 1975; Shamoo and MacLennan, 1974). Antibody studies showed that the 30K fragment is accessible, whereas the 20K fragment is poorly exposed (MacLennan, 1975). The 100K dalton (Ca^{2+}, Mg^{2+}) -ATPase, the 55K dalton and 20K dalton fragments are all shown by Shamoo and MacLennan (1974) to exhibit ionophoric activity by increasing black lipid membrane conductance. The 20K fragment is a calcium specific

ionophore as it requires Ca^{2+} ions for expression of its ionophoric properties and has a selectivity sequence for divalent cations, consistent with the selectivity of transport in intact sarcoplasmic reticulum (Shamoo et al., 1977a,b). In addition, ruthenium red and mercuric chloride which are inhibitors of transport in the intact system, inhibit the ionophoric activity of the 55K and 20K fragments, while methylmercury, an inhibitor of the ATPase, does not inhibit the ionophoric activity (Shamoo and MacLennan, 1975). The above information led Shamoo et al. (1977a) to postulate that the (Ca^{2+}, Mg^{2+}) -ATPase consists of a hydrophobic channel (45K fragment) spanning the membrane. The hydrophilic, 55K fragment, portion located on the exterior cytoplasmic side contains both the 30K dalton phosphorylation site and the 20K dalton ionophoric site. These workers further postulated that the 20K dalton fragment acts as a "gate" as it is partially buried in the hydrophobic interior at the head of the 45K dalton fragment.

The concept of the ATPase being composed of a number of domains with distinct functions has also been suggested by Berman et al. (1977) and McIntosh and Berman (1978) from studies of "uncoupling" of calcium transport from ATPase activity in the presence of mild acid conditions and with EGTA. This uncoupling of calcium transport brought about by inactivation of transport causes a minor perturbation of the ATPase and has the characteristics of a partial denaturation of the ATPase (see Section 1.3).

Trypsin treatment does not cleave other proteins of the intact sarcoplasmic reticulum. The molecular weight of the tryptic fragments and of the high affinity Ca^{2+} binding protein (M_{55}) and calsequestrin (MW 45K) are identical. However, the possibility that the latter proteins are degradation products of the ATPase has been ruled out for several reasons: (a) the tryptic fragments are not water soluble whilst the acid proteins are; (b) the proteins have different amino acid compositions; (c) antibodies raised against the various proteins do not cross react; and (d) the ATPase can be completely digested with trypsin while the acid proteins are unaffected and can be isolated intact from the digested membrane (Stewart and MacLennan, 1974).

1.3 REACTION MECHANISM OF THE (Ca^{2+}, Mg^{2+}) -ATPase

The membrane of the sarcoplasmic reticulum is simple in composition and this makes the study of the only known physiological functions of this membrane, calcium transport and release, relatively straight forward. The (Ca^{2+}, Mg^{2+}) -ATPase of sarcoplasmic reticulum serves as a carrier which is responsible for translocating calcium across the membrane against a concentration gradient. This is an energy-requiring process and the energy is provided by the Ca^{2+}-dependent hydrolysis of ATP. This section deals with the mechanism of "coupling" of ATP hydrolysis to calcium transport.

Calcium accumulation by SR vesicles is coupled stoichiometrically to ATP hydrolysis by membrane bound (Ca^{2+}, Mg^{2+})

-ATPase. Hydrolysis of ATP and simultaneous translocation of calcium into the vesicles involves the transfer of the γ-phosphate of ATP to the enzyme to form an acid-stable phosphorylated enzyme intermediate (EP) (Makinose, 1969). Formation of the phosphorylated intermediate requires both Ca^{2+} and Mg^{2+} whereas hydrolysis of EP is activated by Mg^{2+} but inhibited by high concentrations of calcium (Meissner, 1973).

There are two types of ATP hydrolysis exhibited by SR membrane - Ca^{2+}-dependent (extra) and Ca^{2+}-independent (basal) both having different characteristics (Hasselbach and Makinose, 1961 and 1962). Mg^{2+} is required for full activation of both "extra" and "basal" ATPase activities. Compared to the "extra" activity, the "basal" ATPase activity is independent of Ca^{2+}, has less specific substrate requirements (Makinose and The, 1965), a higher K_m for ATP (Weber et al., 1966; Yamamoto and Tonomura, 1967) and a different dependence on temperature (Inesi et al., 1976) and pH (Yamamoto and Tonomura, 1967). When the SR membrane is solubilized by detergents, the"basal"ATPase activity is abolished (McFarland and Inesi, 1970; Walter and Hasselbach, 1973). The "extra" ATPase activity is extremely calcium dependent between the concentrations 0.01 and 1μM, is abolished by thiol reagents (Hasselbach and Seraydarian, 1966; Inesi et al., 1967; Murphy, 1976) and enhanced during inactivation of transport at low pH (Berman, et al., 1977). It is uncertain whether the two activities can be ascribed to different enzymes (Martonosi, 1964; Seraydarian and Mommaerts,

1965) or may represent interconvertible conformations of the same enzyme (Froehlich and Taylor, 1976; Inesi et al., 1976). Studies on the transient-state kinetic properties of the ATPase by Froehlich and Taylor (1976), showed an early burst of Pi release for the "basal" ATPase which was not a feature of the "extra" ATPase, suggesting that the enzyme-substrate complex does not form the obligatory E~P intermediate, but breaks down directly to E.P, an acid-labile phosphate intermediate. They therefore proposed an alternate pathway of the transport enzyme for the "basal" ATPase activity. Inesi et al. (1976) reported that in SR fractions, having identical protein compositions but different densities, the ratio of the "extra" to "basal" ATPase activities varied. This ratio was also highly temperature dependent ("extra" : "basal" : 9.0 at 40°C and 0.5 at 4°C).

Ca^{2+}-dependent ATP-hydrolysis, also termed "extra ATP splitting" by Hasselbach and Makinose (1961), is tightly coupled to the accumulation of Ca^{2+} by the membrane vesicles. The stoichiometry between Ca^{2+} uptake and ATP hydrolysis, in intact vesicles, in the presence of oxalate under a variety of conditions was found to be 2 mol of Ca^{2+} transported per mol of ATP hydrolysed (Hasselbach and Makinose, 1961 and 1963; Martonosi and Feretos, 1964). This ratio was maintained in the absence of oxalate over a wide range of ATP concentrations (Weber et al., 1966). In transient state kinetics the coupling ratio of 2 mol of Ca^{2+} transported per mol of ATP hydrolysed existed as shown by Kurzmack and Inesi (1977) using rapid mixing and quenching techniques. SR vesicles, treated with organic solvents, i.e. diethylether (Fiehn and Hasselbach, 1969; Inesi

et al., 1967), low concentrations of detergents, i.e. Triton x-100 (McFarland and Inesi, 1970; Walter and Hasselbach, 1973), phospholipases (Fiehn and Hasselbach, 1970; Martonosi and Fortier, 1974) or with EGTA at alkaline pH (Duggan and Martonosi, 1970) were inactivated with respect to calcium transport due to an enhanced "leakiness" of vesicles to calcium. Net uptake of Ca^{2+} by the vesicles was thus prevented by the action of these detergents on the lipid components of the membrane, however the enzyme still retained a high rate of Ca^{2+}-dependent ATPase hydrolysis. It should, however, be noted that it has recently been shown by Salama and Scarpa (1980), that diethylether (5%;w/v) actually enhances the rate of both Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity and does not inhibit Ca^{2+} accumulation as previously described (Fiehn and Hasselbach, 1969; Inesi et al., 1967). This inhibition which was thought to be due to enhanced "leakiness" of the vesicle was proposed by Salama and Scarpa (1980) to be an artifact in the experimental procedures. In some previous experiments, the SR vesicles could have been irreversibly damaged by a combination of the following three steps: incubation of the vesicles in diethylether, then centrifugation, which takes a long time and resuspension of the insoluble material. In other experiments, ATP dependent Ca^{2+} uptake was measured radiochemically through the distribution of $^{45}Ca^{2+}$ in the SR and in the reaction mixture after Millipore filtration. In the presence of diethylether, the characteristics of the filter are altered and the trapping capacity of the filter is reduced. A "true" uncoupling of calcium transport from

ATPase activity has been shown by Rossi et al. (1979) using low substrate concentrations. In the presence of p-nitrophenyl-phosphate, as a substrate, the Ca^{2+} transport decreased as the pH was increased from 6.4 to 7.6. The maximal rate of substrate hydrolysis, however, remained the same. Berman et al. (1977) and McIntosh and Berman (1978) showed a similar "uncoupling" under mild acid conditions at 37°C and in the presence of millimolar concentrations of EGTA at neutral pH and at 37°C respectively. These conditions irreversibly inactivated calcium transport, while Ca^{2+}-stimulated ATPase acitivity was enhanced. These SR vesicles were not rendered permeable to inulin and there did not appear to be any loss of protein. Calcium binding to the high affinity site appeared to protect against this inactivation of transport (McIntosh and Berman, 1978). The characteristics of these inactivations, including stabilization of transport through high affinity calcium binding sites and large entropic and enthalpic contributions was proposed by these authors to possibly be due to a minor conformational change in the (Ca^{2+}, Mg^{2+}) -ATPase. The uncoupling has also been obtained by x-irradiation of SR vesicles (McConnell et al., Radiation Research (1981) in press) and by treatment with halothane (Diamond and Berman, 1979).

Other nucleosidetriphosphates (NTP), besides ATP can support calcium transport by the (Ca^{2+}, Mg^{2+}) -ATPase (Makinose and The, 1965). The velocity of Ca^{2+} transport and "extra" NTP hydrolysis compare to ATP hydrolysis as follows:

ATP (1.0) > ITP (0.8) > GTP (0.7) > CTP (0.6) > UTP (0.3). The coupling ratio of 2 mol of Ca^{2+} ion transported for each mole of NTP hydrolysed is similar for each substrate. The rates of hydrolysis of the nucleotides are related to the affinity of the enzyme for these substrates (de Meis and de Mello, 1973). MacLennan (1970) showed that the purified ATPase hydrolyses UTP, GTP and ITP at rates of 13-16% the rate of ATP. Other phosphate compounds such as acetylphosphate (de Meis, 1969), p-nitrophenylphosphate (Inesi, 1971) and carbamylphosphate (Pucell and Martonosi, 1971) support calcium transport by hydrolysis and phosphorylation of the enzyme. Although 2 mol of Ca^{2+} ions are transported by the extra splitting of one mol of phosphate compound, the rates of their hydrolysis are extremely slow. It appears therefore that a number of artificial substrates, some of which are structurally unrelated to ATP are hydrolysed by the (Ca^{2+}, Mg^{2+}) -ATPase of SR and promote Ca^{2+} transport. The reaction mechanism, including the formation of identical phosphorylated intermediates appear to be similar.

Calcium and magnesium ions are required for full activation of the Ca^{2+}-dependent ATPase activity by SR. Half maximal activation of the enzyme, in the presence of equimolar and saturating concentrations of Mg^{2+} and ATP occurs at low concentrations of calcium ($K_{0.5}$ = 0.3-0.5 μM Ca^{2+}) (Weber et al., 1966). Activation of the enzyme by Ca^{2+} shows non-Michaelis-Menten kinetics with a Hill coefficient of 1.8. This value is consistent with the molar ratio of coupling between Ca^{2+} transport and ATP hydrolysis of 2 and suggests the binding of 2 mol of Ca^{2+} is involved in

the activation (The and Hasselbach, 1972). Calcium concentrations above 0.1mM inhibit ATP hydrolysis (Yamamoto and Tonomura, 1967; Inesi et al., 1970; Makinose, 1969). Other metal ions, which substitute for Ca^{2+}, but with much lower affinity, are Sr^{2+} (Weber et al. 1966; Yamada and Tonomura, 1972) and Co^{2+} (MacLennan, 1970). The enzyme also had a high affinity for the La^{3+} (Yamada and Tonomura, 1972).

There are two different requirements for Mg^{2+} in ATP hydrolysis by SR vesicles. One is to form an equimolar complex with ATP, MgATP, the true substrate (Vianna, 1975) for the Ca^{2+}-dependent ATPase. The other role of Mg^{2+}, is in the catalytic cycle, (Inesi et al., 1967) and involves accelerating the decomposition of the phosphorylated intermediate formed during the hydrolysis of ATP. The ATP-dependence of ATPase activity shows non-Michaelis-Menten kinetics in the concentration range 0.1 to 100 μM MgATP, with a Hill coefficient of 0.3-0.5 showing negative cooperativity (Inesi et al., 1967; Neet and Green, 1977). At approximately 100 μM apparent saturation of this phase occurs. Millimolar concentrations of MgATP cause further activation. Kinetic studies of these two processes by Dupont (1977) and Shigekawa et al. (1978) indicate a high affinity catalytic site and a low affinity regulatory site, which will be discussed in detail below.

Formation of Phosphoprotein as an Intermediate in the ATPase Reaction

In the presence of Ca^{2+} and Mg^{2+}, sarcoplasmic reticulum membranes catalyse a phosphate exchange between ADP and ATP (Ebashi and Lipman, 1962; Hasselbach and Makinose, 1962). It was therefore postulated that a high-energy phosphoprotein was formed as a reaction intermediate in active calcium transport (Hasselbach, 1964). Quenching the Ca^{2+}-dependent reaction of SR membranes with [γ-^{32}P]ATP by TCA resulted in the formation of a phosphorylated protein (Yamamoto and Tonomura, 1967 and 1968; Makinose, 1969; Inesi et al., 1976; Inesi and Almendares, 1968; Martonosi, 1967 and 1969a). This phosphoprotein was shown to be a true reaction intermediate by similarities in (a) Ca^{2+} and ATP dependence of the ATPase reaction and of steady-state phosphoenzyme formation and (b) pH profile of these parameters (Yamamoto and Tonomura, 1968). The phosphoprotein is acid stable, but extremely labile at alkaline pH and is hydrolysed by treatment with hydroxylamine (Makinose, 1969; Yamamoto and Tonomura, 1967 and 1968). These properties indicate that the phosphoprotein is an acyl-phosphate similar to the acyl-phosphoprotein intermediate of the (Na^+, K^+)-dependent ATPase (Post et al., 1972; Skou, 1971). This phosphoprotein is formed by covalent linkage of the phosphoryl group to the β-carboxyl group of aspartate. This was confirmed by Degani and Boyer (1973) who reported that reductive cleavage of the acyl-phosphate bond of the phosphoprotein by $NaBH_4$ yielded

homoserine after acid hydrolysis of the protein. The tripeptide sequence of the phosphorylation site was shown to be (Ser or Thr)-Asp-Lys (Bastide et al., 1973)

Mechanism of the ATPase Reaction

The following scheme shows the steps involved in the molecular mechanism of coupling of the ATPase reaction and the vectorial transport of calcium ions across the membrane of SR, which includes binding, translocation and release of calcium ions (Kanazawa et al., 1971).

$$2\ Ca^{2+}_{(out)} + E + ATP \rightleftharpoons Ca_{2(out)}.E.ATP$$

$$Ca_{2(out)}.E.ATP \xrightleftharpoons{Mg^{2+}} Ca_{2(out)}.E \sim P + ADP$$

$$Ca_{2(out)}.E \sim P \rightleftharpoons 2Ca^{2+}_{(in)} + E - P$$

$$E - P \xrightleftharpoons{Mg^{2+}} E + Pi$$

where $Ca^{2+}_{(out)}$ and $Ca^{2+}_{(in)}$ refer to Ca^{2+} outside and inside the SR vesicles, respectively.

The first step of calcium transport by the (Ca^{2+}, Mg^{2+}) -ATPase involves the random binding, at the outer surface of the membrane, of 2 moles of Ca^{2+} and 1 mol of ATP to 1 mol of ATPase (E), to form the Michaelis complex $E^{Ca_2}_{ATP}$. This leads to the formation of a phosphorylated intermediate, E ~ P which occurs very rapidly on exterior of the membrane. Kinetic studies of Yamada and Tonomura (1972a) and the equilibrium-dialysis method of Chevallier and Butow (1971) and Meissner et al. (1973) indicate that the enzyme has high affinity

Ca^{2+} binding sites (K_D = 0.3μM), nearly 2 mol of Ca^{+} binding sites/mol of ATPase. K_D value of Ca^{2+} obtained by kinetic studies (Yamada and Tonomura, 1972a) are independent of the presence of ATP. Binding of ATP (Meissner, 1973) as well as a non-hydrolysable ATP analogue, β, γ-methylene adenosine 5'-triphosphate (AMPPCP) (Pang and Briggs, 1977) also appears to be unaffected by low concentrations of Ca^{2+}, indicating that initial binding of ligands is in a random sequence. Some reports indicate that the binding may not be random. The Ca^{2+}-dependent hydrolysis of other substrates such as p-nitrophenyl-phosphate (Inesi, 1971) and acetylphosphate (Pucell and Martonosi, 1971) is different from that of ATP. Also, at low concentrations of ATP, alkali metal ions compete with Ca^{2+} for the Ca^{2+} binding sites thus inhibiting transport (de Meis, 1971). This inhibitory effect was decreased by higher concentrations of ATP (> 20 μM). Increasing the concentrations of ATP, during steady-state analysis of ATPase activity, causes a decrease in the K_m for Ca^{2+}, the V_{max}, however, is unaltered (Yamamato and Tonomura, 1967). It was suggested that the higher concentrations of ATP induce a conformational change in the enzyme with an enhanced affinity for Ca^{2+}. Acetylphosphate shows similar trends (de Meis and Hasselbach, 1971).

The Ca^{2+} transport ATPase appears to undergo a conformational change on binding of both Ca^{2+} and ATP to the enzyme. Coan and Inesi (1976, 1977) detected a modification of the electron spin resonance spectrum upon binding of Ca^{2+} and either ATP or acetylphosphate to vesicles previously labelled with the spin label 2, 2, 6, 6-tetramethyl, 4-amino (N-iodo-

acetamide). These results conflicted with results previously obtained using spin labels that react with sulphydryl groups. Previously, similar spectral effects on the addition of ATP were noted by Landgraf and Inesi (1969), Inesi and Landgraf (1970), Pang et al. (1974), Tonomura and Morales (1974), but these studies resulted in conflicting reports as to the requirement for Ca^{2+}, Mg^{2+} and nucleotide. Champeil et al. (1976) found that Ca^{2+} alone induced changes in the spectrum of iodoacetamide analogue spin label (N-(1-oxyl-2, 2, 2, 6-tetramethyl-4-piperidinyl)-iodoacetamide) (ISL) attached to sulphydryl groups on the ATPase. A later report by Champeil et al. (1978), using the spin label ISL, indicated that some of the discrepancies may be due to the fact that different -SH groups are selectively alkylated according to the labelling medium. They confirmed the Ca^{2+}-induced changes of the spectra in the absence of nucleotide. Dupont (1976) and Dupont and Leigh (1978) have shown that the binding of Ca^{2+} to the sites of high affinity of the ATPase resulted in an increase of the intrinsic fluorescence of the enzyme. The rate of the signal change was too slow to represent directly the binding of calcium to the enzyme. A two step process was therefore proposed in which fast binding to the enzyme would be followed by a slow isomerization of the enzyme to a form that had a higher fluorescence. Evidence of Ca^{2+}-induced changes in the conformation of the ATPase was reported by Murphy (1976, 1978) who monitored -SH group reactivities using 5, 5'-dithiobis (2-nitrobenzoate) (DTNB). Calcium binding to the high affinity sites induced a conformation change by

converting three classes of -SH groups to one class displaying a single rate constant (Murphy, 1976). The presence of both Ca^{2+} and adenosine 5'-(β,γ-imino)triphosphate (AMPPNP), a non-hydrolysable analogue of ATP, resulted in a complex with a single set of sulphydryl groups with a reactivity of about half that of the complex with Ca^{2+} alone (Murphy, 1978). Thorley-Lawson and Green (1977) and Andersen and Møller (1977) detected a modification of reactivity of sulphydryl groups only in the presence of both ATP and Ca^{2+}. The use of -SH group reactivity as a probe of conformation in the (Ca^{2+}, Mg^{2+}) -ATPase will be discussed in detail in Section 1.5. Further evidence for Ca^{2+} altering the conformation of the ATPase was obtained by McIntosh and Berman (1978) by measuring the calcium stabilization of the transport system. Low concentrations of Ca^{2+} protected the enzyme against acid and thermal inactivation of Ca^{2+} transport without inhibiting ATPase activity. The conformational change induced by Ca^{2+} binding was shown to be a two-step process of binding and then stabilization. From these data it is concluded that a conformational change in the (Ca^{2+}, Mg^{2+}) -ATPase is promoted by either the simple binding of Ca^{2+} or of both Ca^{2+} and substrate to the enzyme. As far as can be seen from the literature, at present, there is no direct evidence for a conformational change associated with the translocation of Ca^{2+} across the membrane.

EP Formation from E and ATP

Binding of Ca^{2+} and ATP to the (Ca^{2+}, Mg^{2+}) -ATPase of SR, in the presence of Mg^{2+}, results in the formation of the phosphorylated intermediate (Makinose, 1969; Martonosi, 1969a; Inesi et al., 1970; Yamamoto and Tonomura, 1968). Immediately after the addition of low concentrations of ATP to SR in the presence of Ca^{2+}, EP rapidly forms without a lag phase and reaches a steady-state level within 1 second at 15°C (Martonosi et al., 1974; Kanazawa et al., 1971). The absence of a lag phase in EP formation, even in the presence of an extremely low concentration of ATP, which is membrane impermeable (Weber et al., 1966), suggests that the binding of ATP to the ATPase site to form EP occurs very rapidly at the outer surface of the the membrane. An initial lag phase is present on liberation of Pi (Kanazawa et al., 1971), which coincides with the rapid increase in EP formation. $E^{32}P$ formation can be terminated immediately by the addition of EGTA which chelates the calcium in the medium, or if the radioactive ATP is diluted with a large amount of unlabelled ATP. Sarcoplasmic reticulum membranes are impermeable to ATP and EGTA and this indicates that Ca^{2+} and ATP interact with the ATPase enzyme to form the Michaelis complex at the outer surface of the membrane.

The role of Mg^{2+} in formation of the phosphorylated intermediate has produced conflicting results. (Kanazawa et al. (1971) and Makinose (1969) showed that addition of Mg^{2+} was an absolute requirement for phosphorylation. The level of EP was demonstrated to increase in the presence of Mg^{2+}

by Inesi et al. (1970). However, Martonosi (1969a) reported that Mg^{2+} had no effect on the amount of EP formed. These discrepancies may possibly be due to the different methods of isolation employed in which media contain varying amounts of contaminating Mg^{2+}. The requirements for Mg^{2+} may also vary according to whether the initial rate of EP formation or the steady-state concentration of EP is measured. Addition of high concentrations of Mg^{2+} (15mM) was shown by Kanazawa et al. (1971) to have no effect on the steady-state reaction, whereas the initial rate of EP formation was stimulated approximately sixfold. Mg^{2+} stimulates the decomposition of EP (Yamamoto, 1972) and this complicates investigations into the Mg^{2+} requirement of the steady-state EP levels. Therefore, in the presence of high concentrations of Mg^{2+}, an increase of EP, measured in the steady-state, could be masked by an enhanced decomposition of EP. If this were true, high concentrations of Mg^{2+} should stimulate ATPase activity in the steady-state. However, the ATPase activity has been reported to be unaffected (Yamamoto and Tonomura, 1967) or inhibited (Shigekawa et al., 1978) by these high concentrations of Mg^{2+}. EP formation was optimal when the ratio of ATP : Mg^{2+} was one. A similar ratio was found for Ca^{2+} uptake, optimal ATP hydrolysis and ADP $\rightleftharpoons$ ATP exchange (Yamamoto, 1972), suggesting that the requirement for Mg^{2+} in EP formation is due to MgATP being the true substrate of the reaction.

Micromolar concentrations of Ca^{2+} are an absolute requirement for the formation of significant levels of

phosphoenzyme. Ca^{2+}-dependent phosphoenzyme formation coincides with that of calcium transport, Ca^{2+}-dependent ATPase activity and ATP $\rightleftharpoons$ ADP exchange. Unlike ATP hydrolysis, high concentrations of Ca^{2+} (>0.1 mM) do not inhibit EP formation (Makinose, 1969; Inesi et al., 1970; Yamamoto and Tonomura, 1967).

The levels of phosphoenzyme depend on ATP concentration. At 100μM ATP, maximal levels of EP are obtained (Verjovski-Almeida et al., 1978). The measured maximal levels of phosphoprotein range from 1-4 μmols/g protein in the presence of ATP and high concentrations of Ca^{2+} ($\geq$ 100μM) (Froehlichand Taylor, 1975; Verjovski-Almeida et al., 1978; Kanazawa et al., 1971 and Inesi et al., 1970). However, the initial rate of EP formation shows biphasic dependence on ATP concentration. At $0^{o}C$ the initial rate of EP formation increases with increasing ATP concentrations up to 100μM and a further increase in rates of EP formation above this concentration is obtained (Kanazawa et al., 1971). This transition in V_{max} and K_m was similar to that of the ATP dependence of ATP hydrolysis (Weber et al., 1966; Yamamoto and Tonomura, 1967; Inesi et al., 1970). At high ATP concentrations ($\geq$ 100μM) and higher temperatures (20-25^{o}C), the initial rate of EP formation is too rapid to measure accurately (Froehlich and Taylor, 1976; Verjovski-Almeida et al., 1978). However, the first order rate constant of EP formation has been estimated to be 150 s^{-1} or more at $20^{o}C$ (Froehlich and Taylor, 1975) and 85 s^{-1} at $25^{o}C$ (Verjovski-Almeida et al., 1978). These values are based on the ATP-

dependence of EP formation at lower ATP concentrations and maximal phosphoenzyme levels.

Translocation and Release of Calcium

The process of calcium transport across the SR membrane involves binding of Ca^{2+} outside the vesicle, translocation and release of Ca^{2+} inside the vesicle. Ca^{2+} translocated from the outside to the inside of the vesicles is coupled to EP formation. The formation of EP is accompanied by a marked decrease in the affinity of the enzyme for calcium, thus causing the release of calcium in the interior of the vesicle. It was thus postulated that the Ca^{2+} affinity of the ATPase should be different at the external and internal surface of the membrane (Kanazawa et al., 1971; Kanazawa and Boyer, 1973; Ikemoto, 1975; Carvalho et al., 1976; Yamamoto and Tonomura, 1976; Shigekawa and Dougherty, 1978). The external surface of the membrane should have a high affinity site for Ca^{2+} in order to be able to bind this ion even when the Ca^{2+} concentration in the assay medium is less than 10^{-6}M. The SR ATPase contains at least two different classes of Ca^{2+} binding sites, which can be distinguished by their respective affinities. K_s = 0.3-2μM and 0.6-1mM at pH 7.0 (Ikemoto, 1974, 1975; Chevallier and Butow, 1971; Meissner, 1973; Fiehn and Migala, 1971). Ikemoto (1975, 1976) reported that the site of high affinity is converted to a site of low affinity during the process of ATP hydrolysis. It was shown by Ikemoto (1974) that Ca^{2+} bound to the higher affinity sites of the purified

ATPase, that did not accumulate calcium, was released into the medium on addition of a small amount of ATP. After hydrolysis of the added ATP, the released Ca^{2+} was rebound back to the enzyme. The transient-state kinetics of the formation of EP and calcium release was analysed by Ikemoto (1976) by means of rapid-mixing, acid-quenching and by a stopped-flow technique, using Arsenazo III, a Ca^{2+} indicator dye. It was found that on mixing the enzyme with ATP, EP formation occurred immediately, however, the release of Ca^{2+} began only after a significant delay. These findings were explained by sequential formation of two acid-stable phosphorylated intermediates having different affinities for Ca^{2+}. One phosphorylated intermediate, having a higher affinity for Ca^{2+}, was formed immediately after the addition of ATP. This high-affinity intermediate was then transformed to one with lower affinity, thereby allowing release of the bound Ca^{2+}. It has also been shown by Makinose (1973) and Sumida and Tonomura (1974) that Ca^{2+} is translocated and released before dephosphorylation of the enzyme. Inesi and coworkers (Inesi *et al.*, 1978; Verjovski-Almeida *et al.*, 1978) investigated the initial translocation of calcium induced by phosphorylation of the ATPase using a rapid-mixing system with an EGTA quench. An initial rapid translocation of Ca^{2+} (6-7nmol Ca^{2+}/mg protein) took place. This was, however, too fast to be resolved by this system (< 20ms). This process was shown to be dependent on phosphorylation, since substitution of ATP with a non-hydrolysable ATP analogue, adenyl-5^1-yl-imido diphosphate, was ineffective.

Decomposition of EP

The release of calcium into the vesicular lumen, following translocation from outside to inside the membrane, is related to the decomposition of EP. The first order rate constant of $E^{32}P$ decomposition can be measured directly after its formation, by addition of EGTA to remove Ca^{2+}, or by addition of unlabelled ATP to dilute the [γ-^{32}P]-ATP thereby halting ^{32}P incorporation into the enzyme (Martonosi et al., 1974; Inesi et al., 1970; Yamamoto and Tonomura, 1967). The time course of decomposition follows first order kinetics without exhibiting a lag phase (Kanazawa et al., 1971). The initial rate of phosphoenzyme decay at 5°C, initiated approximately 650 m sec after adding ATP by chelation of Ca^{2+} with excess EGTA, was measured by Martonosi et al. (1974). They reported a rate of constant of 0.365 sec^{-1}. This value is 2-3 orders of magnitude slower than phosphorylation and may thus be a rate limiting step in the catalytic cycle. They also showed that delipidation of SR vesicles, by treatment with phospholipases, caused a decrease in the rate of EP decomposition, compared to control vesicles (rate constant of 0.068 sec^{-1} compared with 0.365 sec^{-1} for control vesicles). This treatment did not affect the rate of EP formation and it was therefore suggested that phospholipids are required for EP decomposition, but not for EP formation.

Magnesium is required for the decomposition of EP and chelation of Mg^{2+} by addition of EDTA resulted in a marked inhibition in the rate of EP decay (Martonosi, 1969b;

Kanazawa et al., 1971; Inesi et al., 1970; Panet et al., 1971). The decomposition of EP did not stop immediately on removal of Mg^{2+}, but 5 sec elapsed before decomposition was completely prevented (Kanazawa et al., 1971). Decomposition of EP was terminated immediately on addition of EDTA to SR membranes solubilized with Triton X-100, indicating that dephosphorylation was stimulated by Mg^{2+} at the interior of the vesicle (Kanazawa et al., 1971). EP hydrolysis was reactivated by addition of Mg^{2+} to the EDTA-inhibited system.

High concentrations of calcium (> 0.1 mM) inhibit dephosphorylation by competing at the site where Mg^{2+} accelerated the decomposition of EP. Phosphorylation of the enzyme was unaffected at these Ca^{2+} concentrations (Martonosi, 1969a). Calcium, bound to low affinity Ca^{2+} sites, inhibited ATP hydrolysis and EP decay but promoted the ATP$\rightleftharpoons P_i$ reaction (de Meis and Carvalho, 1974; de Meis and Sorenson, 1975). Yamada and Tonomura (1972a) found that in the phosphorylated form the enzyme has 2.5 fold higher affinity for Ca^{2+}, compared with Mg^{2+}, and suggested that EP could not distinguish between these 2 divalent cations. On the other hand, in the unphosphorylated enzyme, the affinity for Ca^{2+} is about 30 000 fold higher than that for Mg^{2+} (Yamada and Tonomura, 1972a).

Garrahan et al. (1976) have examined the effects of Mg^{2+} on EP decomposition. EP formed in SR vesicles, whose exchangeable Mg^{2+} was removed by washing SR vesicles with trans-1,2-diamino cyclohexonetetraacetic acid (CDTA), did not decompose on addition of an excess of Mg^{2+}. However, EP formed in the presence of Mg^{2+} did decompose when Mg^{2+} was removed by

addition of CDTA. Similar results were found in SR vesicles solubilized with Triton X-100. From these findings, Garrahan et al. (1976) suggested that the Ca^{2+}-binding sites required for EP formation were distinct from the Mg^{2+}-binding site required for the decomposition of EP. They also suggested that Mg^{2+} combined with the ATPase prior to phosphorylation and that phosphorylation caused a conformational change in the protein which resulted in the occlusion of the site at which Mg^{2+} combines to accelerate the decomposition of EP. The exact role of Mg^{2+} in controlling decomposition of EP remains to be elucidated. SR vesicles contain between 7-9 μmol Mg^{2+}/g SR protein which is equivalent to approximately 0.9-1.1 mol mg^{2+}/mol of ATPase (assuming that the ATPase has a molecular weight of approx. 100 000 (MacLennan, 1970) and constitutes 80% of SR protein (Malan et al., 1975)).

Reversal of the Ca^{2+} Pump

The reversal of the entire process of Ca^{2+} transport was first demonstrated by Makinose and Hasselbach (1971); Barlogie et al. (1971); Makinose (1971, 1972) who showed that the Ca^{2+}-dependent ATPase can catalyse the synthesis of ATP from ADP and Pi using the electrochemical osmotic energy derived from a Ca^{2+} gradient formed across the membrane. This reversal was demonstrated using two different experimental approaches - firstly the net synthesis of ATP, coupled with Ca^{2+} efflux from the vesicle (Barlogie et al., 1971; Makinose, 1971; Makinose and Hasselbach, 1971) and

secondly the ATP $\rightleftharpoons$ Pi exchange coupled with Ca^{2+} exchange between Ca^{2+} pools contained in the vesicle and in the assay medium (Makinose, 1971; Makinose, 1973). When vesicles, previously loaded with Ca^{2+}, are incubated in a medium containing EGTA, Ca^{2+} flows out of the vesicle at a slow rate due to the low Ca^{2+} permeability of the membrane. Addition of ADP, Pi and Mg^{2+} to the incubation medium causes a sharp increase in calcium efflux, which is not observed if one of these reactants is omitted from the medium (Barlogie et al., 1971). The fast efflux of calcium is coupled with ATP synthesis (Makinose and Hasselbach, 1971). Inhibition of the fast efflux of Ca^{2+} is observed when the ionic calcium concentrations in the medium are identical to those that activate Ca^{2+} uptake and ATP hydrolysis. Barlogie et al. (1971) thus suggested that the inhibition occurs on the external surface of the vesicle at the same site where Ca^{2+} transport is activated and thus the membrane components involved in active uptake of Ca^{2+} are also responsible for Ca^{2+} efflux. ATP synthesis is initiated by phosphorylation of the Ca^{2+}-dependent ATPase by Pi forming an acylphosphoprotein (E ~ P). Two to four µmoles of phosphoenzyme/g of protein are formed by incubating vesicles, previously loaded with Ca^{2+} in a medium containing EGTA, Pi and Mg^{2+} (Makinose, 1972; Yamada et al., 1972; Yamada and Tonomura, 1973). Phosphorylation of the enzyme by Pi is not accompanied by an increase in Ca^{2+} efflux. Subsequent addition of ADP to the medium leads to a decrease of the steady-state level of phosphoprotein, synthesis of ATP and an increase in the rate of Ca^{2+} efflux (de Meis, 1976). Transfer of the

phosphate from the phosphoenzyme to ADP triggers the fast release.

Panet and Selinger (1972), de Meis (1976) and Punzengruber et al. (1978) showed that in the presence of a Ca^{2+} gradient, SR membranes are phosphorylated by Pi to form phosphoprotein to the extent of 1-3nmol/mg protein.

Makinose (1971) observed that incubation of SR vesicles in a medium containing Ca^{2+}, ATP, ADP, Mg^{2+}, and ^{32}P, resulted in accumulation of Ca^{2+} by the vesicles and formation of a Ca^{2+} concentration gradient until a steady-state was reached in which Ca^{2+} efflux was balanced by the ATP-driven Ca^{2+} influx. When this condition was reached, a steady-state exchange between ^{32}Pi and γ-phosphate of ATP was observed. Vesicles, rendered leaky by phospholipase A treatment, did not support ATP $\rightleftharpoons$ Pi exchange. A low rate of ATP $\rightleftharpoons$ Pi exchange is catalysed by vesicles in the absence of a Ca^{2+} gradient, provided that the low affinity Ca^{2+} binding sites (apparent K_m = 1-3 mM at pH 7.0) which are located on the inner surface of the membrane, are saturated (de Meis and Carvalho, 1974). A decrease in affinity of the ATPase for Pi ($\simeq$ 10 fold) in the absence of a Ca^{2+} concentration gradient accounts for the diminished exchange rate.

The Pi incorporated into the phosphoprotein and ATP is derived from the Pi in the medium and not from the Pi inside the vesicle (de Meis and Carvalho, 1976). ATP (or ITP) competitively inhibit the phosphorylation of SR by Pi, suggesting that phosphorylation by Pi and ATP occur at the same site on the ATPase at the outer surface of the membrane (de Meis and Masuda, 1974; de Meis, 1976).

Mg^{2+} is required for reversal of the pump. ATP synthesis and Ca^{2+} efflux from loaded SR vesicles are not activated by ADP and Pi in the presence of EDTA, a chelator of Mg^{2+} and Ca^{2+} (Hasselbach, 1978). The Mg^{2+} effect is saturated at a Mg^{2+} concentration of 0.1mM.

Verjovski-Almeida et al. (1978) studied the kinetics of phosphoenzyme formation with Pi, using passively loaded vesicles, in the millisecond time scale. Addition of EGTA induced phosphorylation which exhibited an initial lag phase (90ms), followed by a linear rise to an asymptote at 2nmol EP/mg protein. This lag phase was suggested to be due to the conversion of a form of the enzyme with a high affinity for Ca^{2+} and unable to be phosphorylated by Pi to a form with a low affinity for Ca^{2+} and which could be readily phosphorylated. Addition of excess calcium to the medium can disrupt the steady-state levels of phosphoprotein maintained with Pi in the presence of a Ca^{2+} gradient (Verjovski-Almeida et al., 1978).

ADP can only be phosphorylated by EP, formed from Pi, when the low affinity binding sites on the inside of the membranes are occupied. This phenomenon was shown by Beil et al. (1977), by following ATP formation in the presence and absence of a Ca^{2+} gradient and by de Meis and Tume (1977) by subjecting the ATPase to a pH jump. At a low pH (5.5), the high and low affinity Ca^{2+} binding sites were both unoccupied (Verjovski-Almeida and de Meis, 1977). By increasing the pH, the affinities of both sites for Ca^{2+} was increased resulting in ATP synthesis. Thus occupancy of the low affinity site is necessary for formation of ATP by transfer of Pi from EP to ADP (de Meis and Tume, 1977).

Pi ⇌ HOH Exchange and Phosphorylation of the Ca^{2+}-Dependent ATPase by Pi in the Absence of a Ca^{2+} Concentration Gradient

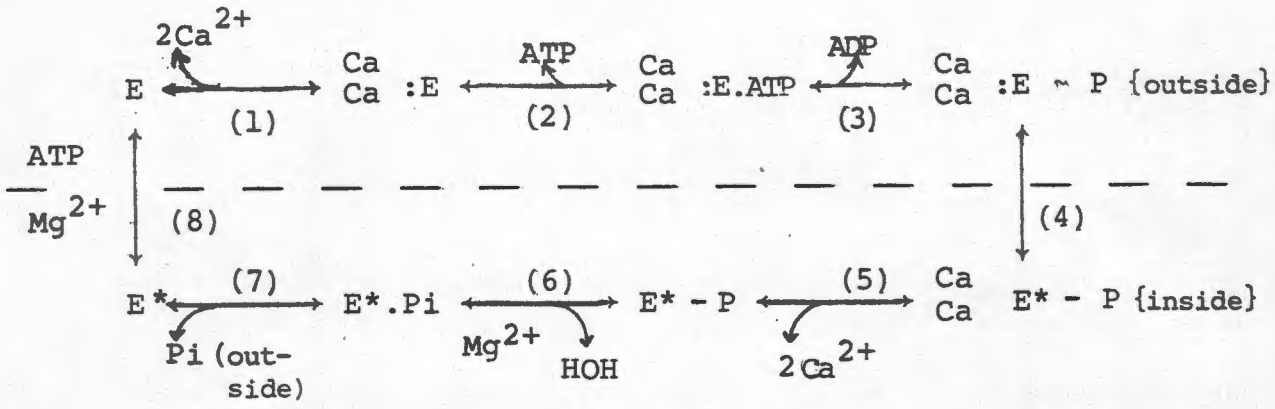
SR vesicles, not previously loaded with Ca^{2+}, were able to catalyse a rapid incorporation of water oxygen atoms into Pi when incubated in the presence of EGTA (Kanazawa and Boyer, 1973). This Pi ⇌ HOH exchange suggested that phosphorylation of the enzyme by Pi was accompanied by the elimination of water. Water oxygen could be incorporated into phosphate oxygen by reversing this reaction. Kanazawa and Boyer (1973) showed that in vesicles, not previously loaded with Ca^{2+}, a small but significant fraction of the enzyme was phosphorylated by Pi. The level of phosphoenzyme measured was 1/50 of the level of phosphoenzyme measured by Makinose (1972) and Yamada et al. (1972) who used vesicles which were previously loaded with Ca^{2+}. Addition of Ca^{2+} concentrations, similar to those required for activation of ATP hydrolysis (1-10μM), to the medium, resulted in inhibition of both the Pi ⇌ HOH exchange and the phosphorylation by Pi. It is apparent, therefore, that Ca^{2+}, bound to high affinity Ca^{2+} binding site, inhibits phosphorylation with Pi. However, phosphorylation by ATP is facilitated under these conditions.

SR membranes can also be phosphorylated by Pi in the absence of ATP or a Ca^{2+} concentration gradient (Kanazawa and Boyer, 1973; Kanazawa, 1975; Masuda and de Meis, 1973). This was shown using vesicles which were rendered leaky, causing dissipation of the concentration gradient. Under optimal conditions, the maximum phosphoprotein formation is in the range

3-4n moles/mg SR protein (Masuda and de Meis, 1973; Punzengruber et al., 1978). The phosphorylation by Pi is inhibited by low concentrations of Ca^{2+} (with half maximal inhibition of EP occurring at 5-10μM Ca^{2+}), Na^{+} and K^{+} (K_i = 100mM) and by both ATP and ADP. Raising the Pi concentration of the medium caused a decrease in the inhibition produced by ADP and ATP, indicating that this is a competitive process (de Meis, 1976; Masuda and de Meis, 1973). Masuda and de Meis (1973) concluded that both ATP and Pi are substrates of the Ca^{2+}-dependent ATPase, which undergoes a conformational change, depending on the binding of Ca^{2+} to a site of high affinity. The preference of Pi or ATP as a substrate for the phosphorylation reaction is determined by the binding of Ca^{2+} to the enzyme. The Ca^{2+} binding sites are located on the outer surface of the vesicles. This was established by the fact that the inhibition of phosphorylation by Pi in the presence of increasing Ca^{2+} concentrations in the medium, is essentially the same whether leaking vesicles or vesicles previously loaded with Ca^{2+} are used. Thus phosphorylation is independent of Ca^{2+} concentration inside the vesicle (Masuda and de Meis, 1973).

Reaction Sequence - A Model for Transport

A minimal reaction scheme has been proposed independently by Carvalho et al. (1976) and de Meis and Boyer (1978) for substrate hydrolysis and Ca^{2+} transport using the partial reactions discussed above.



The enzyme is represented by two distinct functional states E and E*. The E form faces the outer surface of the vesicle and has a high affinity for Ca^{2+} (apparent K_m = 1-3μM at pH 7.0). In the E* from the Ca^{2+} binding site faces the inner surface of the vesicle and has a low affinity for Ca^{2+} (apparent K_m = 1-3 mM at pH 7.0). The E form is phosphorylated by ATP but not by Pi, while the form E* is phosphorylated by Pi but not by ATP. The rate limiting step of the reaction is (8) depending on the nucleotide triphosphate (NTP) used. In the absence of NTP the conversion of E* to E occurs slowly and increasing concentrations of NTP activates the rate of interconversion. ITP is much less effective than ATP in stimulating this reaction. All the reactions in the above scheme are reversible. In the forward reaction, step (4) involves the translocation of Ca^{2+} from the outside to the inside of the membrane. The free energy for translocation is obtained from the high-energy acyl phosphoprotein. A conformational change occurs with phosphorylation of the enzyme resulting in a reduction of affinity of the enzyme for Ca^{2+} and Ca^{2+} is thereby released into the vesicular lumen. The E* form reverts slowly to the E form (reaction 8) after

rapid dephosphorylation of E*-P which has a low chemical potential (reactions (6) and (7)).

Reversal of the cycle occurs when the concentration of Ca^{2+} in the medium is very low and the high affinity Ca^{2+}-binding sites are unoccupied. The enzyme E* can now be phosphorylated by Pi. E*-P is a low energy phosphoenzyme and can only transfer Pi to ADP to synthesize ATP if Ca^{2+} is bound to the low affinity sites.

The above discussion indicates that the overall catalytic cycle of the calcium pump of sarcoplasmic reticulum is complex. However, a number of discrete intermediates and reactions have been well characterised. From the point of view of the present investigation which is concerned with the mechanism of coupling and of uncoupling of the calcium pump, the reaction

$$E - P_{out}^{Ca^{2+}} \longleftrightarrow E^* - P_{in}^{Ca^{2+}}$$

is the most relevant since during this step, a major conformational change in the enzyme occurs which is associated with translocation of calcium against the concentration gradient.

1.4 KINETIC REACTIVITY OF THIOL GROUPS AS A PROBE OF PROTEIN CONFORMATION

The kinetic reactivity of thiol groups has been used extensively as a probe of conformation of enzymes. Thiol groups, which are distinguished from other functional groups of proteins by their high reactivity, enter into many, and often very characteristic and selective, reactions with several types of specific reagents. Sulphydryl and disulphide groups are often involved in specific functions of enzymes and other biologically active proteins and may thus partake in a number of physiological processes. The varied functions of -SH and -S-S groups in enzymes can consist of binding substrates and cofactors, in direct participation in the catalytic act, or in maintaining the native, catalytically active conformation of the protein. The role of these groups in particular functions of specific enzymes will be discussed in this section. Disulphide groups have a more limited reactivity in comparison to sulphydryl groups. The resistance of these groups to various influences and the stability of the S-S bond correspond well with the function of the disulphide groups in proteins, which is mainly in stabilizing the macromolecular structure.

The -SH groups, in most proteins, vary in reactivity. They have been divided into three classes by Hellerman *et al.* (1943) and Barron (1951) : rapidly reacting, sluggishly reacting and "masked" or "buried" -SH groups. The latter group can be detected only after denaturation of the protein and destruction

of its secondary and tertiary structure. The activity of some enzymes, for example, succinate dehydrogenase and glyceraldehyde -3- phosphate dehydrogenase, is inhibited by blocking of rapidly reacting -SH groups. However, inhibition of the activity of other enzymes, for example, urease, aldolase and malate dehydrogenase, is initiated only when their slowly reacting or even "masked" -SH groups are blocked. The class of -SH groups required for enzymatic activity can be established by stepwise titration of -SH groups and parallel determination of enzymatic activity. In aldolase, blocking of the 8-12 most reactive -SH groups (2-3 per subunit) with p-mercuribenzoate did not affect the activity of the enzyme. However, the blocking of these groups produced conformational changes in the enzyme that rendered other -SH groups, which were unreactive or "buried" in the native protein, accessible to the -SH probe. Reaction of these newly exposed groups with p-mercuribenzoate led to further and greater changes in the conformation of the molecules and to loss of enzymatic activity (Szabolcsi and Biszku, 1961; Szajari et al., 1970; Závodszky et al., 1972).

Thiol groups in proteins may be situated in hydrophobic or apolar environments and are thus unavailable in the native enzyme for reaction with a wide variety of specific reagents. However, by increasing the length of the N-alkyl group of the -SH reagent, groups that were originally inaccessible become reactive. The rate of reaction of -SH groups of yeast alcohol dehydrogenase and of kidney D-amino acid oxidase with a series of N-alkylmaleimides increases steeply with lengthening of the

N-alkyl group of the reagent (Heitz et al., 1968; Fonda and Anderson, 1969). The reaction rate of thiol groups of turnip yellow mosaic virus with n-alkyl mercuric nitrates increases with increase in the alkyl chain length (Godschalk and Veldstra, 1965). The rate of reaction of p-mercuribenzoate with the thiol groups of fumerase also increases in the presence of normal aliphatic alcohols, both with the alcohol concentration and with the number of methylene groups in the alcohol (Robinson et al., 1967). These observations indicate the apolar character of the environment of the -SH groups, which evidently assists the binding of reagents that contain the longer alkyl chains. Impermeant maleimides with varying connecting arm lengths have been used to determine the number of -SH groups in different membrane compartments of human erythrocyte membranes (Abbot and Schachter, 1976). These authors showed that, by the use of these reagents, the outer, inner, and interior compartments of the membrane contain approximately 3%, 57% and 40% of the total membrane -SH groups, respectively.

Sulphydryl groups of enzymes are usually divided into essential and non-essential groups. The essential groups are those whose blocking or destruction by various reagents is accompanied by a change in catalytic activity, usually by a loss or decrease, but more rarely by an increase. The role of these groups is usually in the active site of enzymes, taking part in catalysis. They may, however, be situated outside the active site, but participating in the maintenance of an active conformation. The essential -SH groups may also

be part of the allosteric or regulatory site. The non-essential -SH groups, whose blocking *in vitro* does not affect the catalytic activity, may be important for the functioning of the enzyme in the cell. Many enzymes lose activity on chemical modification of the thiol groups, but this is generally insufficient evidence for assigning a mechanistic significance to the -SH group involved. A new group introduced at a sulphydryl position may eliminate or reduce the binding of substrates to the enzyme or the catalytic functions of the enzyme either directly, due to the introduction of a different charge (Jacobson and Stark, 1973) or on account of the steric bulk of the introduced group (Kress *et al.*, 1966; Chung *et al.*, 1971; Smith *et al.*, 1975). An inhibitor molecule, bound to an -SH group, may sterically hinder the access of substrate to the active site, thereby inhibiting enzymatic activity, even though this -SH group itself plays no part in substrate binding or in catalysis. For example, Kress *et al.* (1966) found that the degree of inhibition of muscle ATP-AMP-phosphotransferase (myokinase) by organomercurial compounds increased with enlargement of the reagent molecules; ethyl mercury chloride, phenylmercury chloride, and p-acetamido phenyl mercury acetate inhibited the enzyme by 22, 55 and 75%, respectively. Chung *et al.* (1971) showed the significance of the size and charge of the reagent molecule by reaction of the one most reactive -SH group of isocitrate dehydrogenase from *Azotobacter vinelandii*. Blocking of this group with iodoacetate, p-mercuribenzoate, NEM or DTNB led to an almost complete loss of catalytic

activity. However, replacement of the nitrobenzoate group from DTNB, by cyanide, with formation of a thiocyanoalanyl residue, led to regeneration of 30-50% of the activity. The thiocyanate group is uncharged and less bulky than the above-mentioned reagents and thus apparently allows substrates to bind to the active site of the enzyme. Similarly, oxidation of the -SH group of aspartate transcarbamylase is accompanied by loss of catalytic activity and lowered affinity for substrate analogues (Benisek, 1971). However, conversion of this -SH group into -S-Me or -SCN does not result in loss of activity and binding of substrates is not inhibited (Jacobson and Stark, 1973).

Myosin, isolated under conditions which minimize disulphide bond formation, contains approximately 40 free -SH groups/4.7×10^5 daltons (Buttkus, 1971). Of these approximately 30 are located in the globular head regions, which possess ATP hydrolytic activity, and 10 are located in the helical tail (Huszar and Elzinga, 1971; Lowey et al., 1969). Reaction of the two fastest -SH groups ($-SH_1$) of the heads, in the absence of ligands, with NEM, resulted in an enhancement of Ca^{2+} and Mg^{2+} activities to a maximum level and almost complete abolishment of the K^+ (EDTA) activity (Schaub et al., 1975). Reaction of a further two -SH groups ($-SH_2$) resulted in complete loss of both activities. The same biphasic response to -SH reagents occurred when myosin was treated with p-chloromercuribenzoate (Kielley and Bradley, 1956), phenylmercuric acetate (Perry and Cotterill, 1965) and iodoacetamide (Trotta et al., 1968). The fact that

modification of these specific -SH groups eliminated ATPase activity and because, conversely, the binding of ligands seemed to affect the reactivities of the $-SH_1$ and $-SH_2$ groups towards sulphydryl reagents (Reisler et al., 1974; Schaub et al., 1975), it has been assumed that these two groups of sulphydryl are 1) at the site catalysing ATP hydrolysis (Burke et al., 1973; Reisler et al., 1974) and 2) essential for this activity (Young, 1969; Mannherz and Goody, 1976). Wiedner et al. (1978) submitted evidence that myosin activity can be retained after blocking all -SH groups, provided the blocking group is small, (e.g. cyanide). Even when only 0.6 of the total of 40 -SH group/molecule is free, the enzyme retained elevated levels of Ca^{2+}- and Mg^{2+}- ATPase activity and approximately 4% of its K^{+}-ATPase activity. The enzyme was also protected against NEM inactivation. The authors thus concluded that the sulphydryl groups are not directly involved in the mechanism of ATP hydrolysis by myosin and are thus non-essential.

Stoichiometric incorporation of a small CH_3S- group onto the active -SH group of rabbit muscle creatine kinase led to an enzyme with 18 ±2% residual catalytic activity (Smith and Kenyon, 1974). In contrast, complete inhibition of enzymatic activity with stoichiometric incorporation of a wide variety of common thiol blocking reagents has been reported (Watts, 1973). Incorporation of negatively charged thiol groups appears to have the greatest inhibitory effect on the activity of the enzyme. Iodoacetate delivers the completely inhibitory, negatively charged carboxymethyl group to the active essential -SH group of creatine kinase (Cys-1) (O'Sullivan and Cohn, 1966).

This carboxymethyl group is sterically smaller than a $CH_3CH_2CH_2S$- group for which 8% residual activity is observed (Smith et al., 1975). Tetrathionate, which also delivers a negatively charged group, leads to complete inhibition of creatine kinase (Kassab et al., 1968).

Papain contains only one free -SH group per molecule. This is an essential -SH group and is required for enzymatic activity (Glaser and Smith, 1971). Modification of this group with the relatively small NC- group (Degani et al., 1970) and 1:1 incorporation of a CH_3S- group (Smith et al., 1975) led to total inhibition of enzymatic activity. Rabbit muscle glyceraldehyde -3- phosphate dehydrogenase contains eight reactive -SH groups per mole of enzyme (2 per subunit) (Moore and Fenselau, 1972). Four (one per subunit) have been found to be more reactive to -SH blocking agents. The most reactive sulphydryl group, Cys-149, has been postulated to be essential to the catalytic mechanism (Perham and Harris, 1963; Smith et al., 1975). Glyceraldehyde -3- phosphate dehydrogenase and papain are two of the few enzymes in which direct participation of -SH groups in catalysis has been unambiguously demonstrated. The -SH groups in the dehydrogenase are involved in the formation of a thioester bond with the acyl group of the substrate molecule (Boyer and Segal, 1954; Racker and Krimsky, 1952, 1958). The -SH group of papain plays a similar role of accepting the acyl part of the substrate molecule. Some examples of interactions of -SH groups of enzymes and the carbonyl group of substrates with the formation of enzyme-substrate compounds of the

hemimercaptal or thioester type are shown in the following enzymes: glutamic semialdehyde reductase (Smith and Greenberg, 1957); yeast pyruvate decarboxylase (Schellenberger, 1967), 3-α-hydroxysteroid dehydrogenase (Tomkins, 1956), ribulose-1,5-diphosphate carboxylase (Racker and Krimsky, 1958; Rabin and Trown, 1964) and acetoacetyl-CoA thiolase (Gehring et al., 1968).

Further examples of enzymes where -SH groups are essential for catalytic activity are given by the following examples. Sulphydryl residues of heart muscle succinate dehydrogenase are also shown to be required for the catalytic activity of the enzyme. They perform an essential and not a secondary role in catalysis. These thiol groups, at the active site, may also be the site of tight binding of oxalacetate during the activation-deactivation of the enzyme. This assumption is made by the fact that tightly bound oxalacetate prevents inhibition by NEM and alkylation of the -SH residue(s) at the active site prevents the binding of labelled oxalacetate (Kenney, 1975).

All the available thiol groups of rabbit skeletal muscle pyruvate kinase can be modified with methanethiosulphonate. This correlated with 4 -SH groups per protomer. In the presence of Mg^{2+} alone or with Mg^{2+} and ATP together, only three $-SC^{3}H_{3}$ groups are incorporated into each subunit. The modification of three -SH groups per protomer has a minor effect on enzyme activity, however, the modification of a fourth group makes these the enzyme catalytically inactive. This shows that only a single thiol participates in the

catalytic event and is also important for maintenance of enzyme conformation (Bloxham et al., 1978).

Sulphydryl groups at the active sites of enzymes besides participating directly in the catalytic act, may be involved in establishing bonds between the molecules of substrate or coenzyme. One of the most common methods used to define the participation of -SH groups in substrate or cofactors binding, is to investigate the ability of substrates, coenzymes and their analogues to protect -SH groups from blocking by thiol reagents. A decrease in the number of titratable -SH groups of an enzyme, in the presence of substrate or coenzyme, can be taken as a preliminary indication of the presence of -SH groups in the active site.

In studying the -SH groups of pig heart aspartate aminotransferase, it was found that one -SH group is located near the substrate-binding site of the enzyme (Torchinskii, 1964; Torchinskii and Sinitsyna, 1970). Selective blocking of this group with p-mercuribenzoate (after prior blocking of two non-essential -SH groups) inhibits the activity of the enzyme by 95% and greatly diminishes its affinity for substrates and substrate analogues. This -SH group is alkylated by NEM only in the presence of substrates, with an accompanying large fall in activity (Birchmeier and Christen, 1971). These authors suggest that the induced accessibility of this group reflects a transient conformational change in the enzyme in the course of the catalytic cycle. However, this -SH group plays no essential role in catalysis or in the maintenance of

the active conformation of the aminotransferase.

The kinetic reactivity of sulphydryl groups in a variety of enzymes and the effect of substrates on the -SH reactivity has been widely studied. The following examples exhibit how the rate of reaction of -SH groups can be used to detect the different conformations of proteins by distinguishing different classes of -SH groups that exist in an enzyme. Tryptophan-transfer ribonucleic acid synthetase contains 8 -SH groups of which 3 react very rapidly and the remaining 5 at a slower rate. In urea-denatured protein, 8 -SH groups are also reactive, the same number as in the native enzyme. This indicates the absence of buried -SH groups. In the presence of tryptophan and ATP, the 3 fast-reacting -SH groups are still accessible, however 4 of the 5 slow-reacting groups are no longer detected (DeLuca and McElroy, 1966).

Guidotti (1965) showed the difference in the conformation of oxyhaemoglobin and carbon monoxide-haemoglobin by the difference in the velocity of the reaction of -SH groups with DTNB in the two proteins.

The structure and function of rabbit skeletal muscle phosphofructokinase has been elucidated to a large extent by -SH group reactivity. In phosphofructokinase, five kinetic sets of -SH groups have been discerned, one of which contains two groups that become considerably less reactive upon binding of substrates or modulators (Kemp and Forest, 1968). Fluorescence resonance energy transfer has been used to measure

the distance between the cAMP binding site of phosphofructokinase and the most reactive -SH group of the enzyme (Kemp and Forest, 1968). The cAMP site has been labelled with a fluorescent probe which is used as an energy donor to different energy acceptors located at a reactive -SH group. The distance between the cAMP binding site and this reactive -SH group is 28Å (Craig and Hammes, 1980) and the distance between the same -SH group and the citrate binding site is 40Å (Wolfman and Hammes, 1977). This -SH group is situated near a MnATP binding site, which may be catalytic or regulatory (Jones et al., 1973). These results indicated that the ligand binding sites on phosphofructokinase are separated by relatively large distances, which is consistent with the regulatory mechanism being allosteric in nature (Craig and Hammes, 1980).

A kinetic investigation of the reaction of DTNB with -SH groups of rabbit muscle aldolase was helpful in elucidating a number of different properties of the enzyme. The tetrameric structure of the enzyme was confirmed by the fact that four -SH groups per oligomer react much faster with DTNB than any of the others (200 m^{-1} sec^{-1}). The enzyme remains active after this limited reaction. Eight groups per oligomer react with a rate constant of 2.5 m^{-1} sec^{-1}, resulting in inactivation of the enzyme. One of the -SH groups of this group of two -SH groups per subunit can be protected by substrate, preventing inactivation of the enzyme. Five groups per subunit react very slowly or not at all (Gutfreund and McMurray, 1970).

Kinetic reactivity of sulphydryl groups has been used to demonstrate a sulphydryl essential for the deaminase activity of the bifunctional enzyme formimino transferase-cyclodeaminase from porcine liver (Drury and MacKenzie, 1977). This enzyme is composed of eight identical polypeptide chains of about 64 000 daltons, arranged in a circular structure. Each of the eight subunits is a bifunctional polypeptide. The two activities appear independent of one another and it is probable that the two enzymic activities involve separate active sites. The activities have been separated both functionally and physically by selectively inactivating one of the enzyme reactions by means of -SH reagents. The deaminase is sensitive and can be selectively inactivated by chemical modification. Complete loss of the deaminase activity correlates with modification of two -SH groups per subunit, one of which is responsible for the deaminase activity. This -SH group is protected from reaction with DTNB by the inhibitor folic acid and is thus probably at the active site (Drury and MacKenzie, 1977).

Sulphydryl groups play an important role in maintaining the quaternary structure of enzymes and other proteins. Blocking the -SH groups of phosphorylase a with p-mercuribenzoate, methylmercury nitrate or iodoacetamide not only greatly diminishes the enzymatic activity, but also dissociates the enzyme into its four subunits. The original tetrameric structure is restored by subsequent addition of cysteine to the mercaptide of the enzyme (Madsen and Cori, 1956; Madsen,

1956). These authors propose that the cause of inactivation is a rapid change in the protein structure, following blocking of the -SH groups, and that this finally leads to dissociation into subunits. There are a number of other proteins, which dissociate into subunits under the influence of thiol reagents. A few of these are liver glutamate dehydrogenase (Rogers et al., 1962), fumerase (Hill and Kanarek, 1964), formyltetrahydrofolate synthetase (Himes and Rabinowitz, 1962; Nowak and Himes, 1971), liver fatty acid synthetase (Butterworth et al., 1967), yeast hexokinase (Lazarus et al., 1968) liver pyruvate carboxylase (Palacian and Neet, 1970), human haemoglobin (Chiancone et al., 1970), hemerythrin (Duke et al., 1971), glyceraldehyde -3- phosphate dehydrogenase (Smith and Schachman, 1971) and apartate transcarbamylase (Gehart and Schachman, 1965).

Spectrophotometric titration of sulphydryl groups in SDS-denatured bovine heart muscle cAMP-dependent protein kinase with DTNB reveals a total of 16 -SH groups per holoenzyme tetramer, R_2C_2 (mol wt 190 000). In the native holoenzyme, twelve of these -SH groups are accessible for reaction with DTNB. The available -SH groups are distributed four per R subunit (mol wt 55 000) and two per C subunit (mol wt 40 000). Substantial disruption of the quaternary structure occurs after reaction of all 12 -SH groups on the holoenzyme. However, by studying kinetic reactivity of the 12 -SH groups with DTNB, the subunit and substrate interaction relevant to the catalytic and regulatory mechanism of the enzyme can be probed (Armstrong and Kaiser, 1978). These authors demonstrated that binding

of cAMP to the holoenzyme, or isolated R subunit, prevents the reaction of one -SH group per R subunit. However, modification of -SH groups has only a small effect on cAMP binding to R. This suggests that the -SH groups on the holoenzyme, or R subunit, are not directly involved in cAMP binding to the enzyme. The disappearance of one reactive -SH group per R subunit can probably be attributed to a cAMP-induced conformational change in the regulatory subunit of a cAMP-dependent protein kinase. Thiol group modification of the catalytic subunit with DTNB results in > 95% loss of catalytic activity.

α-Crystallin, the multisubunit protein in the eye lens, is a microheterogeneous mixture of apparently spherical molecules with molecular weights ranging from 7×10^5 to 10×10^5 (Spector et al., 1971). Two main types of subunits occur in bovine α-crystallin, the A_2 and B_2 polypeptide chains, which are 57% homologous and are present in a ratio of approx. 3:1 (Van der Ouderaa et al., 1974; Delcour and Papaconstantinou, 1974). The quaternary structure of α-crystallin is unresolved, and it is of interest to know whether all subunits are in (semi-) equivalent positions such as in the spherical shells of viruses, or if there is an arrangement involving exposed and buried subunits. Chemical modification of -SH groups has provided a useful tool in helping to define the structure of α-crystallin. The bovine A_2 chain has only one cysteine residue, whereas B_2 has none at all (Van der Ouderaa et al., 1974). If all A_2 chains are in equivalent positions, then all the -SH groups should show the same kinetic reactivity towards specific thiol reagents. However, Siezen et al. (1978)

demonstrated that at least three classes of -SH groups occur, with various reactivities towards DTNB, 4,4'-dithiopyridine, iodoacetamide and ethylenimine. These authors concluded that the 30 A chains in the α-crystallin molecule are not all in (semi-) equivalent positions, but are arranged in at least three different orientations according to thiol reactivity. Aspartate transcarbamoylase (ATCase) dissociates into separate catalytic (C) and regulatory (R) subunits upon addition of p-mercuribenzoate (Gehart and Schachman, 1965). This allows the study of the separate functions of the subunits of this allosteric enzyme. Gehart and Schachman (1968) found that the -SH groups of the enzyme react with excess p-mercuribenzoate according to pseudo-first-order kinetics and that the rate constant for the reaction of the 24 thiols of the three regulatory subunits is increased sixfold by the addition of both the aspartate analogue, succinate, and substrate, carbamoyl phosphate, which bind to the catalytic subunit. The large increase in the rate of the reaction was interpreted in terms of a conversion of the enzyme molecules from a constrained state, with low affinity for substrates, into a relaxed conformation, having a high affinity for substrates. The authors also proposed that in the presence of succinate and carbamoyl phosphate, the enzyme goes into a "swollen" conformation, in which it more easily dissociates into subunits.

Changes in the conformation of enzymes, brought about by mercaptide-forming reagents, can lead not only to decreases, but also to increases in enzymatic activity. Kielley and Bradley (1956) noted that the addition of a small quantity of

p-mercuribenzoate to myosin in the presence of Ca^{2+} ions increased its ATPase activity 3-4 times. However, increase in reagent concentration led to complete inhibition of the enzymic activity (Petrushkova and Bocharnikova, 1968). Similarly, malate dehydrogenase can be stimulated by Hg^{2+} ions and p-mercuribenzoate (Kuramitsu, 1968; Silverstein and Sulebele, 1970) and glutamate dehydrogenase (Rogers et al., 1962, 1963) and dihydrofolate reductase (Kaufman, 1964) by organic mercury compounds. The allosteric enzymes appear on this list because, in the absence of effector, they are not normally in their most active conformation.

Liver fructose-1, 6-disphosphatase is activated when 5-6 of the 20 -SH groups of the enzyme are modified with p-mercuribenzoate, iodoacetamide, NEM, o-iodosobenzoate and a number of disulphides, such as cystamine, DTNB and diethyl-disulphide (Little et al., 1969). The increase in activity is 220-450% on reaction with disulphide, and about 100% on reaction the other reagents listed. The activated enzyme differed from the native enzyme in solubility and in sensitivity to the action of the allosteric inhibitor, AMP. It is most probable that the cause of activation is conformational changes of the enzyme, whose degree and character depends on the nature of the bound thiol reagent (Pontremoli et al., 1967).

Boyer (1959, 1960) has suggested that the changes in conformation of proteins, that occurs on blocking of their -SH groups, should not be regarded as evidence of direct participation of such groups in forming the structure of the proteins. He emphasized two points, firstly that the major

changes in protein structure occur usually only as a result of blocking slowly reacting on poorly accessible -SH groups. These groups are more important in maintaining protein structure than readily reacting ones, as they may be involved in intramolecular interactions which are destroyed when they are blocked. Secondly, that these changes in protein structure do not occur instantaneously after the -SH groups are blocked, but are delayed. Boyer suggests that the approach of the inhibitor to the -SH groups is sterically hindered and this steric hindrance may be periodically removed during small fluctuations in conformation of the protein. The temporary removal of the barrier makes possible the blocking of the -SH groups and this in turn prevents return of the protein to its original, energetically favourable, and consequently more stable, conformation. Repetition of this process can lead to destabilization of the protein molecule and, finally, to its denaturation.

It appears, therefore, that there are various explanations of the conformational changes that occur in proteins on blocking of their -SH groups. Each individual protein and its reaction with a number of different thiol reagents must be studied carefully to determine whether the -SH groups are important in maintaining the conformation of the protein or whether the -SH group is merely the point of combination of a deforming molecule.

In most enzymes and proteins, -SH group reactivity has been used extensively to probe the conformation of the enzymes and to determine the effect of binding of substrates

and cofactors on the catalytic activity of the enzymes. A few examples have been used in this section to point out the importance of -SH groups in determining the conformation of enzymes. There are, however, many more examples where -SH group reactivity has been used to probe the conformation of different enzyme systems. These have not been mentioned as they require a detailed description of the structure, function and mechanism of action of the enzyme e.g. (Na^+, K^+) -ATPase.

Finally, two interesting examples of where -SH group reactivity has been used to probe conformational changes in proteins are discussed. Spin-label studies of the sulphydryl environment in bovine serum albumin has been used to show different conformations of the protein. Bovine serum albumin is known to undergo several pH-dependent conformational transitions. On the acidic side of the isoelectric point, albumin undergoes the reversible structural isomerization, known as the N-F transition (Aoki and Foster, 1956) and the so-called acid expansion (Yang and Foster, 1954). Sogami and Foster (1968) showed that as the pH is lowered the protein undergoes a fairly cooperative conformation change (N-F transition) that involves a slight expansion of the molecule, resulting from a separation of intramolecular domains and the opening of a crevice. Further decrease of pH causes the protein to undergo a more complete expansion, with a substantial increase in flexibility of the molecular structure. Bovine serum albumin contains 17 disulphide bonds and a single reactive -SH group, located relatively close to the amino-terminal end of the polypeptide chain (King and Spencer, 1972, Brown, 1975).

This -SH group is important for physiological functions (Putnam, 1965) and plays a specific catalytic role in the formation of a disulphide interchanged isomer (Stroupe and Foster, 1973). This -SH group is situated in a crevice approx. 10Å deep (Hull et al., 1975). Cornell and Kaplan (1978a) observed a clear N-F transition, as well as acid expansion, with a spin label shown to project to the lip of the crevice. In the acid-expanded isomer, the -SH group is in a largely exposed environment. These results indicate that the sulphydryl group is in the crevice, formed by the domains of albumin, which opens during the N-F transition. In addition to the acidic transitions, both bovine and human serum albumin undergo a pH-dependent conformational change in slightly alkaline solutions (Leonard et al., 1963). This transition, known as the neutral of N-B transition, involves a cooperative change in the tertiary structure of the molecule, without any significant change in helical content. The N-B and N-F transitions are very similar, in that the -SH group moves from a restricted to unhindered environment during both (Cornell and Kaplan, 1978b). A possible explanation for these observations is that the -SH group is situated in the crevice, which is presumably lined with lone pairs. The molecule undergoes specific conformational transitions as a result of decreasing or increasing the pH, followed by additional unfolding and expansion at more extreme pH. One net effect of all the conformational transitions is the emergence of the -SH group from a restrictive environment to a relatively free one.

The two essential sulphydryl groups of myosin have been studied in detail and some interesting results with regard to the conformation of this protein have emerged. The ability of myosin to hydrolyse ATP is associated with two essential -SH groups, SH_1 and SH_2, in the chymotryptic subfragment I of the molecule. These groups can be specifically modified by thiol reagents. At pH 7.0, in the absence of nucleotides, the SH_1 group reacts with NEM, while SH_2 is practically inaccessible to the reagent (Sekine and Kielley, 1964). However, the SH_2 group becomes modified at a very slow rate if the SH_1-NEM myosin is treated with NEM at pH 7.9. Addition of MgADP to this reaction system dramatically accelerates the rate of modification of the SH_2 group. In the presence of MgADP, SH_2 group moves from a buried to an exposed position (Yamaguchi and Sekine, 1966). Distinctive changes in the ATPase properties of myosin are associated with these sequential modifications of the SH_1 and SH_2 groups. Blocking of the SH_1 group causes elevation in the Ca^{2+} -ATPase and a loss of EDTA-ATPase activities. Complete loss of the ability of the molecule to hydrolyse ATP is accompanied by subsequent blocking of the SH_2 group. The spatial separation of the two essential thiols of myosin is sensitive to the binding of nucleotides. These two critical thiols, SH_1 and SH_2, can be crosslinked by a 12 to 14 Å thiol crosslinking reagent, N, N'-p-phenylenedimaleimide (pPDM) (Reisler et al., 1974). Furthermore, Burke and Reisler (1977) reported that crosslinking with shorter bifunctional thiol reagents occurred optimally when the crosslinking was performed in the presence

of MgADP. Upon binding of MgADP, these -SH groups could move from 14 Å to as close as 7 Å. It has further been proposed that these crosslinked -SH groups can approach to within 3-5 Å of each other, based on evidence of simultaneous chelation of these two -SH groups by a single-exchange-inert Co(111) phenanthroline complex (Wells et al., 1979a,b). Recently, Wells and Yount (1979) noted that magnesium nucleotide stimulated crosslinking of thiols by cobalt phenanthroline complexes or pPDM leads to stable stoichiometric trapping of a 1:1 Mg.nucleotide complex at myosin's active site. It has been proposed that myosin contains a jawlike active site structure, which closes on the Mg.nucleotide complex, bringing together two critical -SH groups to allow crosslinking by cobalt complexes or pPDM (Wells and Yount, 1979). The latest evidence shows that by using DTNB in the presence of MgADP, these -SH groups can approach close enough (2Å) to form a cystine disulphide bond with concommitant trapping of Mg.nucleotide and loss of Ca^{2+}-ATPase activity (Wells and Yount, 1980).

Sulphydryl group reactivity has been extensively used to probe the conformation of enzymes and proteins. This is due to the fact that the functions of -SH groups of proteins are extremely varied. In some enzymes they play a catalytic role, taking a direct part in forming intermediates in the course of the reaction, catalysed by the enzyme. The -SH groups can also participate in the binding of substrates and cofactors to the enzyme. In some cases -SH groups contribute to the stabilization of the catalytically active conformation of the enzyme.

Inhibition of the activity of a number of enzymes by sulphydryl specific reagents is due to destruction of the three-dimensional structure, either as a result of breakage of intramolecular bonds, which involve -SH groups, or by the deforming or destabilizing action of an -SH bound inhibitor on nearby parts of the protein molecule.

Although there remain uncertainties in many instances as to their role, sulphydryl group reactivity of proteins appears to be a reliable and sensitive probe of protein conformation.

1.5 STRUCTURE OF (Ca^{2+}, Mg^{2+}) -ATPase OF SARCOPLASMIC RETICULUM WITH PARTICULAR REFERENCE TO THE SULPHYDRYL GROUPS AND THEIR REACTIVITY

A knowledge of the detailed structure of the (Ca^{2+}, Mg^{2+}) -ATPase and studies of the structure-function relationship will lead to an understanding of the mechanism of this enzyme. The (Ca^{2+}, Mg^{2+}) -ATPase of sarcoplasmic reticulum is a suitable protein for the study of such relationships between structure and function. The protein is a single polypeptide of MW 102 000 (MacLennan et al., 1971) and has been shown to be the active enzyme, since, when incorporated into vesicles composed of a bimolecular layer of phospholipid, the molecule catalyses both ATP hydrolysis and Ca^{2+} transport (Racker, 1972; Warren et al., 1974a). Racker (1972) incorporated the purified (Ca^{2+}, Mg^{2+}) -ATPase of SR into phospholipid vesicles containing Ca^{2+} precipitable anions and he demonstrated that the enzyme catalysed ATP-dependent Ca^{2+} transport. This observation was confirmed by Warren et al. (1975) using a single defined phospholipid. It was thus concluded that the ATPase protein contains, within its polypeptide chain, sites of both Ca^{2+} ionophoric activity and ATP hydrolysis. The mechanism of transduction of free energy between the chemical substrates such as ATP and the vectorial transport of ions across membranes is a fundamental problem in membrane-bound systems. An attempt at understanding this problem has been carried out in the (Ca^{2+}, Mg^{2+}) -ATPase by locating specific regions or fragments of the polypeptide, which possess these distinct functions of ionophoric activity and ATP hydrolytic

activity and by studying the relationship between these two functions. This has been achieved by selectively cleaving the enzyme with trypsin, thereby producing fragments in which these functions can be assayed.

Proteolytic cleavage of the enzyme by trypsin has been discussed in detail in Section 1.2. In the following paragraph, a brief summary of the cleavage products and their functions will be described. The enzyme is cleaved in its native state, to two fragments with molecular weights of 55 000 and 45 000 (Stewart and MacLennan, 1974) by limited tryptic hydrolysis. This cleavage is not accompanied by loss of Ca^{2+} transport function or loss of ATPase activity. At this stage, it has been shown that it is possible to dissolve the fragmented enzyme in SDS solution and to reconstitute Ca^{2+} transport activity after removal of SDS on an anion exchange column (MacLennan et al., 1976). Prolonged digestion results in the cleavage of the 55 000 dalton fragment into 30 000 and 20 000 dalton fragments and in a loss of calcium transport activity. The hydrolytic activity of the enzyme is not affected (Stewart and MacLennan, 1974). The cleaved protein hydrolyses ATP with no loss in ATPase activity. The site of ATP hydrolysis is assayed for by incorporation of ^{32}P from $[\gamma\text{-}^{32}P]$ATP into the peptide chain and by labelling with tritiated N-ethylmaleimide. The radioactive label is found in the 100 000 dalton enzyme and in 55 000 and 30 000 dalton fragments (Stewart et al., 1976; Thorley-Lawson and Green, 1973). The Ca^{2+}-selective ionophoric site, assayed by its ability to increase divalent metal ion conductance in a bimolecular lipid

layer, is isolated in the 55 000 dalton fragment and the 20 000 dalton fragment obtained after cleavage of the 55 000 dalton fragment (Shamoo et al., 1976). The Ca^{2+} dependency and selectivity is not as pronounced in the 20 000 dalton fragment as in the intact enzyme or in the 55 000 dalton fragment (Shamoo, 1978). The relative conductance change and relative permeability elicited by the ionophoric material has the following sequence: $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+}$, Hg^{2+}, Na^{+}, K^{+}, Cs^{+}, Li^{+} and Rb^{+}. Zn^{2+}, Hg^{2+} and Na^{+} strongly inhibit the increase in Ca^{2+} conductance (Shamoo and MacLennan, 1974). The ATPase activity and Ca^{2+} transport are inhibited by methylmercuric chloride and mercuric chloride, whereas the Ca^{2+} ionophoric activity is unaffected by methylmercuric chloride. These substances probably inhibit ATPase and Ca^{2+} transport activities by blocking essential -SH groups (Shamoo and MacLennan, 1975). These authors suggested, however, that there are no essential -SH groups in the Ca^{2+} ionophore and mercuric chloride probably inhibited this Ca^{2+} ionophoric activity by the Hg^{2+} ions competing with the Ca^{2+} ions for the ionophoric site. Digestion of the 55 000 dalton fragment to 30 000 and 20 000 dalton peptides uncouples the two functions, Ca^{2+} uptake being abolished while ATP hydrolysis remains unaffected. The uncoupling indicates that the bond between the 30 000 dalton and 20 000 dalton fragments is essential for energy transduction between the chemical hydrolysis of ATP and the vectorial Ca^{2+} ion transport across the membrane (Shamoo and Murphy, 1979). Berman et al. (1977) have shown a similar uncoupling

of Ca^{2+} transport activity from ATPase activity by pre-treating SR vesicles at low pH and 37°C for a short time. This causes a decrease in Ca^{2+} transport without affecting the ATPase activity which appears slightly enhanced. The ATP-binding site and Ca^{2+}-transporting site appear, therefore, to be distinctly segregrated and thus located on different segments of the polypeptide. A detailed structural study of the ATPase molecule could lead to a clear understanding of the functional relationship between these two distinct sites.

The distribution of sulphydryl groups and disulphide bonds within the ATPase and its tryptic fragments has been determined by Thorley-Lawson and Green (1977). They located the thiol groups using N-ethyl [^{14}C] maleimide or 5,5'-dithiobis-(2 -nitrobenzoate), and showed that the (Ca^{2+}, Mg^{2+}) -ATPase from SR contains 20 -SH groups/ 115 000 daltons in the presence of SDS. After reduction of the enzyme there were 26 -SH groups/ATPase. This value agrees favourably with 26.5 cysteine residues found by amino acid analysis (MacLennan, 1970; Thorley-Lawson and Green, 1975). The difference between this number of thiols and the 20 -SH residues measured before reduction shows that there are approx. six -SH groups involved in disulphide bond formation, giving rise to three disulphide residues. Preparations of (Ca^{2+}, Mg^{2+}) -ATPase made in the absence of dithiothreitol contained three to six fewer -SH groups. The disappearance of thiol groups, during purification in the absence of dithiothreitol, that were not regenerated by reduction, suggests that they were oxidized

to cysteic acid. Reduction with dithiothreitol following purification will regenerate other partially oxidized intermediates. The distribution of exposed and buried thiol groups and of disulphide bonds among the tryptic fragments of the molecule was measured after labelling with ^{14}C-NEM. The tryptic cleavage products obtained by Thorley-Lawson and Green (1973) have the following molecular weights. Single cleavage yields fragments of molecular weight 60 000 (A) and 55 000 (B). Further cleavage of the 60 000 dalton fragment (A) yields fragments of molecular weight 33 000 (A_1) and 24 000 (A_2) (double cleavage). The molecular weights of the fragments differ slightly from those used in this text, which were obtained by Stewart et al. (1976) and thus the above nomenclature (namely, A, B, A_1 and A_2) has been used by Thorley-Lawson and Green (1977) for convenience of reference. The following Table 2 shows the distribution of thiol groups in the tryptic fragments obtained by Thorley-Lawson and Green (1977). The three disulphide bonds are located on fragment B. Most of the thiol groups which become oxidized during the purification of the ATPase, are located on fragment A. The number of buried groups on the tryptic fragments was determined by reacting the exposed groups with NEM and then treating with SDS and labelling the extra exposed groups with ^{14}C-NEM. There appear to be 3.8-4.2 residues labelled. Fragments A and B have equal labelling and therefore each contain two buried thiol groups. Fragments A_1 and A_2 from the doubly cleaved preparations appear to be equally labelled and thus each contain one buried -SH group. However, the presence of background radioactivity in the region of these fragments makes it difficult to make firm conclusions.

TABLE 2: Distribution of Thiol Groups and Disulphide Residues between Tryptic Fragments of (Ca^{2+}, Mg^{2+}) -ATPase (Thorley-Lawson & Green, 1977)

Nature of Preparation of ATPase		Fragment (MW)			
		A 60 000	B 55 000	A_1 33 000	A_2 24 000
ATPase purified in presence of dithiothreitol (non-reduced)	Total thiol groups	13.1	6.8	9.9	3.2
	*Total cysteic acid	13.0	14.0	9.0	3.5
	Disulphide residues (by difference)	0.1	3.6	0.5	0
	Buried thiol groups	2	2	(1)	(1)
ATPase purified in absence of dithiothreitol (non-reduced)	Total thiol groups	7.8	6.2	6.6	1.4
	Thiol groups oxidised during isolation (by difference)	5.3	0.6	3.3	1.8

*Cysteic acid content of the performic acid-oxidized fragments determined previously by Thorley-Lawson and Green (1975).

The reaction of antibodies raised against the fragments indicate that the 55 000 dalton fragment is in large part exposed to the cytoplasm *in vivo*, since, reaction with antibodies raised against the 55 000 dalton fragment is strong. However, the 45 000 dalton fragment is largely buried within the membrane where it associates with phospholipids and reaction with antibodies raised against this fragment is weak. Since some of the antigenic sites of the 45 000 dalton fragment are reactive, part of this fragment must be exposed. It is also unlikely that trypsin would cleave precisely at the interface with the aqueous environment (Stewart *et al.*, 1976). The 30 000 dalton fragment, contains the active site of the ATPase

and is exposed to the cytoplasm, outside the lipid-containing portion of the membrane (Thorley-Lawson and Green, 1973). This conclusion is in agreement with the observations of Hasselbach and Elfvin (1967) who showed, using electron microscopy, that after specifically labelling the active site with azoferritin, the label was clustered at the outer surface of the membrane. An external location for the site of ATP hydrolysis has also been suggested by the use of antibodies (Stewart et al., 1976). The use of antibodies raised against the 20 000 dalton fragment, which contains ionophoric activity, shows that this fragment is located at the external surface, but that it is not fully exposed. This fragment does have a relatively hydrophobic amino acid composition (Stewart et al., 1976). It is important to obtain more knowledge of the chemistry of the ionophoric site and to identify the smallest peptide domain which is responsible for the ionophoric activity. Also of great interest is the location of this site, which is probably responsible for the movement of Ca^{2+} ions from one side of the membrane to the other or which has been postulated to act as a gate, near the external surface, thereby allowing the movement of Ca^{2+} through a membrane channel (Shamoo et al., 1977).

Recently Allen and co-workers, in a series of papers, have established the sequence of nearly three-quarters of the ATPase molecule and this has proved very helpful in aligning the tryptic fragments and localizing the site of ionophoric activity (Allen, 1980a, b ; Allen et al., 1980a,b).

The difficulties of sequence determination arose from the fact that a fraction of peptide material from the protein (approx. one-third) is derived from the interior of the membrane. Allen and co-workers constructed five continuous lengths of amino acid sequence from soluble overlapping peptides obtained from tryptic digestion of the succinylated carboxymethylated protein, α-chymotryptic digestion of the carboxymethylated protein, peptic, thermolytic, tryptic and staphylococcal-proteinase digestion of the reduced protein and digestion of the (Ca^{2+},Mg^{2+})-ATPase with cyanogen bromide. Digestion with the above-mentioned proteinases releases about two-thirds of the protein as soluble peptides. These may be relatively easily isolated by standard techniques. The remaining portion of the molecule is resistant to digestion and gives rise to large, aggregated, relatively hydrophobic peptides, which are insoluble in aqueous solutions and concentrated urea or guanidinium chloride solutions (Allen, 1980a, b; Allen and Green, 1978; Allen *et al.*, 1980a). Most of the cysteine residues are reactive in the native protein (Murphy, 1976; Thorley-Lawson and Green, 1977) and these are released in the soluble peptides. Almost all of the tryptophan residues are close to, or within, the lipid bilayer and are thus not released.

The orientation of the major tryptic fragments of the (Ca^{2+}, Mg^{2+})-ATPase of SR has been proposed by Klip *et al.* (1980) and Tong (1980) by determining the initial amino acid sequences of the isolated fragments and relating these to Allen's five sequences. The alignment of the fragments is

NH_2 - Ac - 20 000 - 30 000 - 45 000 - COOH. The 20 000 dalton fragment has been further subfragmented using cyanogen bromide (CNBr). There are 4 methionine residues in this fragment (Shamoo et al., 1976) and since one methionine is N-terminal, CNBr treatment yields 4 peptides with molecular weights of about 13 000, 7 500, 4 500 and < 1 000 in addition to NH_2-Ac-homoserine derived from the NH_2-terminal Ac-methionine (Klip et al., 1980; MacLennan et al., 1979). Klip et al. (1980) showed that the 13 000 dalton fragment is an end product of the CNBr reaction since it was free of methionine and methionine-sulphone, but contained homoserine. The amino acid composition of this fragment shows that it contains 12 alanine residues, giving a calculated weight of 13 675 which is in good agreement with the value of approx. 13 000 obtained by SDS-gel electrophoresis. Amino acid analysis of the performic acid-oxidized or carboxymethylated protein shows that this fragment contains 2 cysteine residues. The 13 000 dalton fragment has an amino acid terminal sequence identical to that beginning with the second residue of the intact ATPase and runs some 120 amino acids into the chain. This also confirms that the 20 000 dalton fragment is situated at the NH_2 terminus of the (Ca^{2+}, Mg^{2+}) -ATPase of SR. This 13 000 dalton fragment appears to contain hydrophobic regions (Klip et al., 1980).

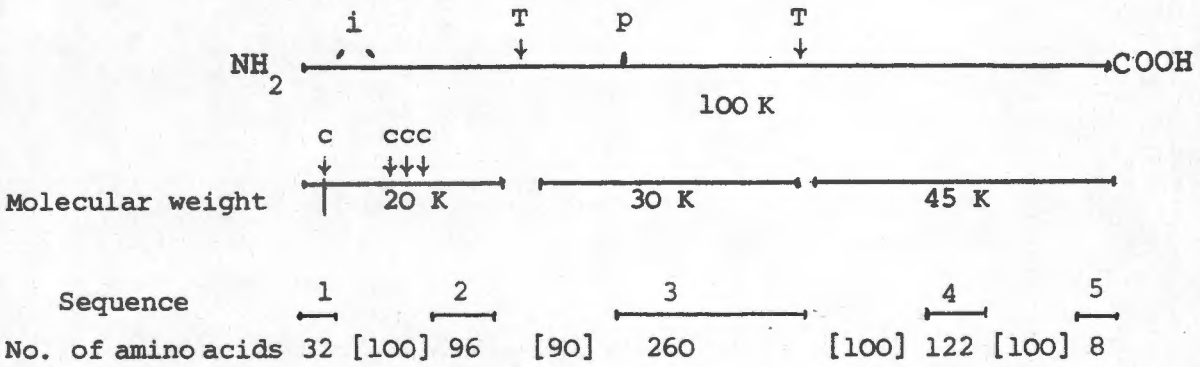
The 7 500 dalton fragment was also shown to be an end product of the CNBr reaction (Klip et al., 1980) since it was free of methionine or methionine-sulphone. This fragment

did not contain homoserine lactone, the product of the CNBr reaction with methionine, and its C-terminal amino acid was arginine. This indicates that this fragment is derived from the COOH terminus of the 20 000 dalton fragment which also has a terminal arginine. This sequence appears to be hydrophilic in nature (Klip et al., 1980). The 4 500 dalton fragment lies proximial to the 7 500 fragment with the 1 000 dalton fragment situated between the 13 000 and 4 500 dalton fragments. The order of alignment of the subfragments of the 20 000 dalton fragment is :- NH_2-Ac-13 000 - 1 000 - 4 500 - 7 500 - (Klip et al, 1980).

The 13 000 dalton fragment has been shown to possess divalent cation ionophoric activity and is several fold selective for cations over anions (MacLennan et al., 1979). The order of selectivity is $Mn^{2+} > Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$. This selectivity sequence is different from that obtained for the 100 000, 55 000 and 20 000 dalton fragments whose selectivity is $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+}$ (Shamoo and MacLennan, 1974). It is assumed that the selectivity is somewhat modified in the 13 000 dalton fragment by the purification procedure (MacLennan et al., 1979). The 7 500 dalton fragment did not appear to have any ionophoric activity and the ionophoric properties of the 4 500 and 1 000 dalton fragments are yet to be determined (MacLennan et al., 1979).

The sequences 1 to 5 of Allen (Allen et al., 1980b) have been aligned by Allen et al. (1980b); Klip et al. (1980) and Tong (1980). Allen has renumbered his sequences as

previously the sequences obtained by Allen (1977) were aligned as follows:- NH_2 - 1 - 3 - 4 - 2 - 5 - COOH. However, Allen has changed his numbering so that the sequences can now be aligned NH_2 - 1 - 2 - 3 - 4 - 5 - COOH. Klip et al. (1980) and Tong (1980) use Allen's old numbering system for the sequences. Scheme 1 shows the alignment of the tryptic fragments with respect to Allens 5 sequences.



SCHEME 1 Alignment of Tryptic Fragments of (Ca^{2+}, Mg^{2+}) -ATPase with Regard to Allen's Sequences 1-5 (Allen et al., 1980b)

where NH_2, NH_2 terminus; COOH, COOH terminus; T, tryptic cleavage sites; c, CNBr cleavage sites; i, ionophoric site; p, phosphorylation site.

Estimates of the number of amino acid residues in unsequenced regions of the polypeptide are given in parentheses.

The 20 000 dalton fragment is situated at the NH_2 terminus of the polypeptide chain. This has been discussed previously with respect to the CNBr cleavage products. The 45 000 dalton fragment is located at the COOH terminus, as

carboxypeptidase Y digestion of this fragment and the ATPase were shown to have identical C-terminal sequences.

The NH_2-terminus of the 30 000 dalton fragment is situated 6 amino acid residues away from the COOH-terminus of Allen's sequence 2. This sequence contains the tryptic cleavage site between the 20 000 and 30 000 dalton fragments which occurs between arginine and alanine residues. Allen's sequence 3 contains the cleavage site between the 30 000 and 45 000 dalton fragments which also occurs between an arginine and alanine bond. It also contains the phosphorylation site at an aspartate residue within the sequence (Scheme 1 p.80). The exact location of sequence 4 is uncertain, however, it contains 122 amino acids, and has thus been assigned to the 45 000 fragment as it is too large to fit in any of the other fragments. Allen's sequences 1 and 5 are at the NH_2 terminus and COOH terminus of the polypeptide chain, respectively. Thus the 20 000-, 30 000 and 45 000- dalton fragments make up the entire (Ca^{2+}, Mg^{2+}) -ATPase molecule.

In order to ascertain the number of transmembrane traverses in each buried segment that has not been sequenced, a knowledge of the location of sequences 1-5 in relation to the surface of the membrane is required. The phosphorylation site, located in sequence 3, is known to be situated on the cytoplasmic face of the molecule (Hasselbach and Elfvin, 1967; Thorley-Lawson and Green, 1973). Sequences 2 and 4 are given similar locations for a number of reasons: (1) a tryptic cleavage site is located in sequence 2 and this implies that it is situated on the cytoplasmic face; (2) all three fragments

are iodinated by lactoperoxidase in the whole SR (Thorley-Lawson and Green, 1973); (3) antibodies raised against the different fragments bind to all fragments even though binding to the 20 000 and 45 000 dalton fragments is weak (Stewart et al., 1976) and (4) the size of negatively stained cytoplasmic projections account for at least half the molecule. This is considerably more than the 260 residues of sequence 3 (Ikemoto et al., 1968; Inesi and Asai, 1968); (5) sequence 4 has 3-SH groups clustered in a very polar region and thus it is probable that this segment, together with the remainder of the water soluble segments of the 45 000 fragments are located on the cytoplasmic face. There is no direct information as yet on the location of the NH_2 terminus and COOH terminus. The maximum number of transmembrane passages would occur if both the N- and C- termini were located on the cytoplasmic surface. A segment of 90 to 100 amino acids would permit a double passage of the peptide through the membrane (Klip et al., 1980). If there were three buried segments this would imply six membrane traverses. However, it is likely that there are four buried segments, two of which are associated with the 45 000 dalton fragment as shown in Scheme 1, p.80. This fragment contains approx. 200 amino acids in water-insoluble fragments and this could permit two separate membrane spanning segments. Thus it is likely that there are eight membrane traverses, derived from two transmembrane passage in the 20 000 dalton fragment, two in the 30 000 dalton fragment and four in the 45 000 dalton fragment. However, there is no direct evidence that each segment penetrates

to the cisternal face and the above argument is based on that assumption.

Allen and Green (1978) previously identified 18 different peptide sequences containing cysteine residues. Of these one is found in sequence 1, none in sequence 2, eleven in sequence 3 and three in sequence 4. The remaining three are released from aggregated peptides by chymotrypsin. Three more can now be added to the list as a result of the sequencing work of Allen and coworkers. These three are located outside the 5 main sequences. There are, however, probably 24 unique cysteine-containing sequences. It is not yet known which cysteine residues form disulphide bonds and which correspond to buried or reactive -SH groups. These known cysteine containing peptide sequences are important, as thiol groups of the ATPase can be selectively labelled in a number of ways (Hasselbach and Seraydarian, 1966; Coan and Inesi, 1977; Thorley-Lawson and Green, 1977) and thus identification of labelled peptide is now possible.

Allen et al. (1980b) suggested some general secondary structural features of the (Ca^{2+}, Mg^{2+}) -ATPase which consist of regions of β-structure that are segregated from the helical regions, thus indicating that any β-sheets appear to be anti-parallel. Also sequences 2 and 3 resemble two predominantly β-structural regions. It should be noted that, at this stage these suggestions are hypothetical.

The (Ca^{2+}, Mg^{2+}) -ATPase exhibits a complex folding pattern in the membrane and thus before being able to understand the mechanism of Ca^{2+} transport across the membrane,

more information on the structure of the ATPase is required. The NH_2 terminal region has a comparatively high percentage of glutamyl residues. The 13 000 dalton fragment situated at the NH_2 terminus contains 17% glutamic acid estimated by amino acid analysis, while the rest of the molecule contains less than 10% (Stewart et al., 1976; Thorley-Lawson and Green, 1975). It is questionable whether or not these glutamyl residues are involved in the formation of the two Ca^{2+} binding sites of the ATPase (Tong, 1980). Kretsinger (1976) has identified a Ca^{2+}-binding loop in a number of Ca^{2+} regulatory proteins. These sites contain several aspartic and glutamic acid residues, alternating with other residues in a characteristic manner and are termed "EF hands". In the 5 sequences of Allen there is no resemblance to amino acid sequences of "EF hands". However, the two Ca^{2+} binding sites of the ATPase function in a totally different manner from those of the regulatory proteins and it is also possible that the peptides responsible for Ca^{2+} binding are associated with the membrane and have thus not yet been isolated. Tong (1980) suggests that it is possible that Ca^{2+} ions may pass through a channel formed from the polypeptide chain or chains and that this channel may be situated in a hydrophobic region, in the 45 000 dalton fragment, about one-third of the molecule away from the COOH-terminus. This region is likely to interact extensively with the membrane as previously shown by Stewart et al. (1976).

In the above discussion, the distribution of thiol groups amongst the (Ca^{2+}, Mg^{2+}) -ATPase and its tryptic subfragments is

described. Most of these cysteines residues are located outside the membrane and appear to be mainly exposed to the surface of the protein. These groups are therefore readily accessible to a variety of thiol-directed reagents and can thus be selectively labelled in a number of ways (Hasselbach and Seraydarian, 1966; Nakamura et al., 1972; Tonomura and Morales,1974; Murphy, 1976; Yoshida and Tonomura, 1976; Coan and Inesi, 1977; Thorley-Lawson and Green, 1977; Yu et al., 1977; Vanderkooi et al., 1977; Ikemoto et al., 1978). The reactivities of -SH groups towards different -SH probes allows the conformational changes, which take place accompanying binding of substrates and ligands to the enzyme to be monitored. This could in turn lead to an elucidation of the partial reactions involved in energy transduction and Ca^{2+} translocation (Murphy, 1978; Coan et al., 1979). Section 1.3 discusses conformational changes in the (Ca^{2+}, Mg^{2+}) -ATPase which occur as a result of binding of ligands and substrates to the enzyme and the conflicting reports arising due to their separate or combined effects on the reactivities of -SH groups. The following discussion relates to the reactivity of -SH groups of the ATPase with particular reference to the conformational states of the enzyme during the functional cycle of the transport protein. Major reorganisation of the membrane protein in relation to Ca^{2+} transport probably does not take place, as suggested by the failure to observe changes the circular dichroism spectrum of the protein during transport (Mommaerts, 1967).

The total number of reactive thiol groups in sarcoplasmic reticulum vesicles varies between 11-14 mol -SH groups/mol ATPase (Hasselbach and Seraydarian, 1966; Panet and Selinger, 1970; Thorley-Lawson and Green, 1977). This number was increased to approximately 20 mol -SH groups/mol ATPase after solubilization of the vesicles in dodecyl sulphate (Thorley-Lawson and Green, 1977). Murphy (1976) found a total of 25 thiol groups/ATPase, 17 of which were reactive. The kinetic reactivity of sulphydryl modification varies according to the different authors. Anderson and MØller (1977) and Hasselbach and Seraydarian (1966) indicated the presence of two classes of reactive thiol groups in the presence of DTNB, with rate constants of 0.17 and 0.015 min^{-1}, respectively, measured at 20°C, pH 7.5 and 0.5 mM DTNB. Murphy (1976) analysed his results in terms of three classes of reactive thiol groups, consisting of 2, 8 and 7 -SH groups per molecule ATPase, respectively. The reactivities of the two fastest reacting thiol groups were approx. 40 times higher than those observed by Hasselbach and Seraydarian (1966). This difference could probably be accounted for by the different conditions used which were higher temperatures (25°C), pH (7.8) and DTNB concentration (4 mM). Thorley-Lawson and Green (1977) found two classes of reactive thiol groups with a stoichiometric ratio of rapidly reacting to slowly reacting sites of approx. 1:4. There were approx. 4 unreactive -SH groups in their preparation of purified SR vesicles. Ikemoto et al. (1978) found three classes of reactive thiol groups in their purified (Ca^{2+}, Mg^{2+}) -ATPase preparations, using the fluorescent reagent

S-mercuric N-dansyl cysteine.

The presence of adenine nucleotides (0.1 mM) protects against the reaction of N-ethylmaleimide with the -SH groups. (Hasselbach and Seraydarian, 1966; Panet and Selinger, 1970) by masking of one -SH group, located near the phosphorylation site. A cysteinyl residue has been located two amino acid residues away from the aspartyl group which is phosphorylated by Mg.ATP (Allen and Green, 1976). Hasselbach and Seraydarian (1966) showed that blocking of four out of ten reactive thiol groups/10^5 g of SR protein with NEM results in complete inhibition of ATPase activity and Ca^{2+} uptake. ATP protects one -SH group and thus prevents loss of ATPase activity and Ca^{2+} transport activity. Yoshida and Tonomura (1976) reported that blocking of at most two thiols with NEM results in complete inhibition of ATPase. Yu et al. (1977) postulated that the -SH groups required for the (Ca^{2+}, Mg^{2+}) -ATPase activity, although located on the external surface of the vesicles, are buried in a restricted microenvironment, related to the tertiary structure of the ATPase and are not related to the phospholipid structure of the membrane. Higher concentrations of ATP (1 mM) appear to decrease the modification rate of slowly reacting thiol groups and this may be attributed to conformation changes of the protein (Panet and Selinger, 1970; Yoshida and Tonomura, 1976; Anderson and Møller, 1977).

High concentrations of Ca^{2+} (1 mM) stimulate the modification of thiol groups of the ATPase. However, this was considered to be non-specific since Mg^{2+} exerted a similar

effect on sulphydryl reactivity (Anderson and Møller, 1977; Thorley-Lawson and Green, 1977). Murphy (1976) however, reported an enhancement of the reaction rate of slowly reacting -SH groups, at concentrations of free Ca^{2+} close to the concentration range where Ca^{2+} is actively transported by the protein in the presence of Mg.ATP. Ca^{2+}-induced conformational changes detected by electron spin resonance (ESR) (Champeil et al., 1976) and fluorescence spectroscopy (Dupont, 1976), have also been reported. It appears, therefore, that Ca^{2+} binding to the high affinity sites on the ATPase induces a conformational change, since phosphorylation of the ATPase by ATP requires Ca^{2+}, while the enzyme may be partially phosphorylated by Pi in the absence of Ca^{2+} and presence of Mg^{2+} (Kanazawa and Boyer, 1973; Knowles and Racker, 1975). Such a conformation change is unlikely, however, to be the basis for Ca^{2+} movement across the membrane.

Phosphorylation of the enzyme in the presence of Ca^{2+}, Mg^{2+} and ATP causes a generalized decrease in the modification rate of slowly reacting -SH groups and an increase in the number of rapidly reacting SH groups per molecule, resulting in a net increase in modification rate during the first minutes of the reaction with DTNB (Anderson and Møller, 1977). The decrease in reaction rate of the slowly reacting -SH groups is consistent with results obtained by Francois (1969), who detected slower exchange of labile hydrogen in Ca^{2+}-transporting vesicles of SR as compared to preparations examined under non-transporting conditions. The results on ascorbate quenching

of nitroxide-labelled -SH groups also suggest a less exposed state of the enzyme during phosphorylation (Tonomura and Morales, 1974).

Interpretation of the above observations is rather limited due to the large number of reactive thiol groups in an ATPase polypeptide (Murphy, 1976; Thorley-Lawson and Green, 1977; Ikemoto et al., 1978) and by the fact that the attachment site of -SH labels has not been characterized in terms of either its location or functional role (Ikemoto et al., 1978; Murphy, 1978). Yamada and Ikemoto (1978) thus studied distinct thiols involved in the specific reaction steps of the (Ca^{2+}, Mg^{2+}) -ATPase by controlling Ca^{2+} concentration during the reaction with NEM. Kawakita et al. (1980) have also characterized the distinct classes of -SH groups of the ATPase molecule with respect to both their reactivity and catalytic function. These authors labelled individual -SH groups which were modified in a specific manner by controlling the NEM concentration and pH of the reaction mixture. At pH 7.0, four distinct thiol groups react with NEM. Two of these are essential for Ca^{2+} transport; one being involved in E-P formation and the other in E-P decomposition. The remaining two -SH groups are non-essential and were shown to be mutually distinguishable by the reactivity towards NEM (Kawakita et al., 1980). These authors obtained specific protection of the -SH group involved in E-P formation by the nonhydrolyzable ATP analogue, AMP-P(NH)P. This proved to be helpful in characterizing individual -SH groups. These results are largely consistent with those of Yamada and

Ikemoto (1978) who showed the existence of two kinds of essential -SH groups whose integrities were required for E-P formation and decomposition, respectively. They also found a highly reactive non-essential -SH group. These 4 reactive -SH groups, out of a total of 24 -SH groups, are distinguished from each other by their kinetic reactivity and are designated by Kawakita et al. (1980) in order of decreasing reactivity as SH_N, SH_D, $SH_{N'}$, SH_F. SH_D and SH_F are responsible for E-P decomposition and formation respectively. SH_D has very similar characteristics to SH_2 reported by Yamada and Ikemoto (1978) and the function of SH_F is the same as that described for S_F by Yamada and Ikemoto (1978). However, S_F is referred to as a group of several kinetically indistinguishable -SH groups, whereas SH_F represents one -SH group, blocking of which results in the loss of E-P forming activity. Kawakita et al. (1980) suggested that SH_F is uniquely located in the vicinity of the ATP-binding site as it was completely protected from reactivity with NEM by AMP-P(NH)P.

S_N, the most reactive -SH group (Kawakita et al., 1980), is not required for Ca^{2+} transport activity and its reactivity is not affected by the presence of Ca^{2+} or nucleotides. The reactivity of SH_D and SH_F towards NEM is, however, markedly affected by the presence of Ca^{2+} ions. A concentration range of 10^{-7} to 10^{-6} M Ca^{2+}, which is similar to that required for activation of E-P formation, causes a marked enhancement of SH_D reactivity. This increased reactivity is probably due to a conformational change of the ATPase,

induced by binding of Ca^{2+} to the high affinity transport site (Kawakita et al., 1980; Yamada and Ikemoto, 1978). Binding of Ca^{2+} to the enzyme also appears to facilitate the interaction of ATP or its analogues with the ATPase, presumably through a conformational change or by relocating these nucleotides in a suitable orientation (Kawakita et al., 1980).

Coan et al. (1979) were able to account for most of the elementary steps in the catalytic mechanism by observing spectral changes, under varying conditions, in the electron spin resonance (ESR) spectra of sarcoplasmic reticulum labelled with an iodoacetamide spin probe (2,2,6,6-tetramethyl 4 amino (N-iodoacetamide)(ISL)) on the -SH groups of the ATPase protein. The sequence of elementary steps involved in the catalytic cycle is given by the following scheme of Coan et al., (1979). These elementary steps were directly implied from the experimental evidence obtained from the spectra.

$$ATP + ADP \cdot E_{out} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ATP \cdot E_{out} + ADP \qquad (1)$$

$$ATP \cdot E_{out} + Ca^{2+}_{out} \overset{K_{Ca_{out}}}{\rightleftharpoons} (ATP \cdot E \cdot Ca_{out}) \qquad (2)$$

$$(ATP \cdot E \cdot Ca_{out}) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} (ADP \cdot E \sim P \cdot Ca_{out}) \qquad (3)$$

$$(ADP \cdot E \sim P \cdot Ca_{out}) \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} ADP \cdot E\text{-}P \cdot Ca_{in} \qquad (4)$$

$$ADP \cdot E\text{-}P \cdot Ca_{in} \overset{K_{Ca_{in}}}{\rightleftharpoons} ADP \cdot E_{in}\text{-}P + Ca^{2+}_{in} \qquad (5)$$

$$ADP \cdot E_{in}\text{-}P + H_2O \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} ADP \cdot E_{in} + Pi \qquad (6)$$

$$ADP \cdot E_{in} \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} ADP \cdot E_{out} \qquad (7)$$

where E_{out} represents the enzyme with high affinity Ca^{2+} binding sites exposed to the outside surface of the membrane and E_{in} the enzyme conformation with low affinity Ca^{2+} binding sites exposed to the inner membrane surface. It appears that the nucleotide remains bound to most of the enzymatic forms contributing to the spectra and thus the authors used the enzyme·nucleotide complex to represent the free enzyme in the above scheme (Coan et al., 1979). Step 1 in the sequence is brought about by simple binding of nucleotide to the enzyme which causes a small initial spectral change. Nucleotide binding alone produces a significant effect on the spectrum in contrast to binding of Ca^{2+} alone which has no apparent effect on the spectrum under the conditions used. When the substrate analogue, AMP-P(NH)P, is used, spectra identical to those of ATP binding are obtained. Under these conditions Ca^{2+} remains bound to the external high affinity sites whether intact or vesicles treated with sonication or Triton X-100 are used (Coan and Inesi, 1977; Inesi et al., 1978b). The binding complex of Step 2 is represented by the spectrum associated with binding of AMP-P(NH)P + Ca^{2+}, to the enzyme, where Ca^{2+} is bound to the high affinity activating sites. There is a specific conformational change in the (Ca^{2+}, Mg^{2+}) -ATPase on binding of Ca^{2+} when nucleotide is also present. It appears that the probe is sensitive to the structure in the immediate vicinity of the nucleotide binding site and that Ca^{2+} binding changes the structural integrity of this site (Coan et al., 1979). Ca^{2+}-induced conformation changes have previously been observed (Champeil et al., 1978; Murphy, 1976, 1978; Dupont, 1976). Steps

2 to 4 occur in rapid sequence (Verjovski and Inesi, 1979; Froehlich and Taylor, 1975) and thus the spectrum observed when Ca^{2+} is added to the ATP·*E* complex must be attributed for the most part to the ADP·*E*-P form of the enzyme (Steps 4 and 5) (Coan et al., 1979). This form of the enzyme is that of the acid-stable phosphorylated intermediate, which is predominant during the high turnover rate steady state. In this form, the spectrum is represented by disappearance of the constrained component and this can be interpreted as a form in which Ca^{2+} has already been released on the inside of the vesicle. Step 5 represents the enzyme where the low affinity internal binding sites are saturated with Ca^{2+}. This can be achieved in the presence of millimolar concentration of Ca^{2+} either by experimental manipulation with vesicles treated with sonication or by Triton X-100 or by accumulation of Ca^{2+} in intact vesicles. The phosphoenzyme then returns to a conformation in which a constrained component appears in the spectrum. The appearance of a constrained component in the spectrum has been shown to follow Ca^{2+} saturation of internal, low affinity binding sites after active transport of Ca^{2+} across the membrane of SR vesicles in the presence of ATP (Coan et al., 1979). These authors have shown that the return of the constrained component, as intravesicular Ca^{2+} increases, is highly cooperative as shown by Scatchard and Hill plots of titration data. Two Ca^{2+}/unit of spectral change are required. A Hill coefficient of 2 is also obtained for the rate of enzymatic inhibition associated with low affinity

binding. In the case of high affinity binding identical spectra and the same high degree of cooperativity is obtained and thus it has been suggested by Coan et al. (1979) that the same Ca^{2+} binding sites and cooperative mechanism may be involved in both Ca^{2+} binding at the high affinity activating sites on the cytoplasmic side of the vesicles and the release of Ca^{2+} inside the vesicle after phosphorylation of the enzyme.

Information relevant to the tertiary structure of the enzyme can be provided by the potential of the cysteine residues for forming crosslinks in the enzyme. Hebdon et al. (1979) showed that there was relatively little intermolecular crosslinking when the more reactive groups were oxidized, in the presence of I_2, even at room temperature. These thiol groups, however, readily form intramolecular disulphide bonds. After cleaving the enzyme with trypsin, it was shown that these disulphides were confined within each major tryptic fragment. Crosslinked molecules or fragments were only observed when the less reactive thiol groups were oxidised (Hebdon et al., 1979). Oligomers of the ATPase, which may be present, are thought to arise by random collisions during the crosslinking reaction (Chyn and Martonosi, 1977; Hebdon et al., 1979). Murphy (1976a) used a crosslinking catalyst, cupric phenanthroline, to form disulphide bridges between ATPase protein chains and he suggested these ATPase units exist as a tetramer within the bilayer. Other workers (Hebdon et al., 1979; Chyn and Martonosi, 1977; Louis et al., 1977) have, however, been unable to repeat the results

obtained by Murphy (1976a). There is a possibility that there may be dimers present in equilibrium with monomers and that these dimers are the species associated with active transport. However, it was shown that the concentration of dimers did not increase under conditions required to bring about translocation of Ca^{2+} (Hebdon et al., 1979). Further information and more convincing evidence is required before an oligomeric structure, based on crosslinking studies, for the ATPase can be accepted.

The study of the sarcoplasmic reticulum is an exciting area of development, since it is the first cationic active pump protein whose primary sequence has been almost entirely determined. This makes it possible to correlate the structure with the known ionophoric and catalytic properties of the enzyme. Sulphydryl groups and their reactivity have played a major role in determining the structure of this enzyme.

2.0 MATERIALS AND METHODS

2.1 PREPARATIVE METHODS

2.1.1 Isolation and Purification of Sarcoplasmic Reticulum Vesicles (SRV)

The method employed was essentially similar to that described previously by Eletr and Inesi (1972) with slight modifications. Sarcoplasmic reticulum was isolated from white skeletal muscle (200 g), excised from the hind legs of a New Zealand white rabbit, crossed with a commercial hybrid strain male. Immediately after excision, the tissue was isolated and cooled in 0.1 mM EDTA pH 7.0. Batches (100 g) of trimmed muscle were homogenized in 400 ml of medium A : 10 mM histidine, 0,3 M sucrose, 0.1 mM EDTA, pH 7.0, 15 sec every 5 min for 1 hr. The pH was kept at 7.0 during this procedure by addition of 5% (w/v) NaOH. The homogenate was centrifuged at 15 000 × g (9 500 r.p.m.) for 20 min. in 250 ml containers in rotor No. GSA in a Sorvall RC-28 centrifuge. The supernatant was collected and filtered through several layers of glass wool, previously washed with medium A to remove low-density lipid aggregates. The filtered suspension was centrifuged at 40 000 x g (20 000 r.p.m.) for 90 min in a Beckman model L2-65B ultracentrifuge using rotor No. 21. The supernatant was discarded and the sediment was resuspended in 100 ml of medium B:10 mM histidine - 0.6 M KCl, pH 7.0 and incubated at 2-4^{o}C for 40 min. The suspension was centrifuged at 15 000 x g (11 000 r.p.m.) for 20 min. in rotor No. 5534 in a Sorvall RC-28 centrifuge. The supernatant was

retained and centrifuged at 78 000 x g (30 000 r.p.m.) for 60 min. rotor No. 30 in a Beckman Spinco model L ultracentrifuge. This final sediment was resuspended in 10 ml of medium C:10mM imidazole, 0.3 M sucrose, pH 7.0. The yield was approx. 4-5 mg SR protein/g of muscle tissue. All the procedures were carried out at 0-4°C. Stock suspensions of SR vesicles (approx. 25 mg protein/ml) were stored for up to 4 days at 0°C. The calcium transport activity remained stable during this period and varied between 1.8 and 2.2 μ mol calcium transported/min/mg protein at 25°C.

2.1.2 Acid and EGTA Inactivation of Calcium Transport by SR Vesicles

Calcium transport activity of SR vesicles, treated with mild acid conditions (Berman et al., 1977) or in the presence of millimolar concentrations of EGTA (McIntosh and Berman, 1978) is inactivated without inhibiting ATPase activity.

For acid inactivation, SR vesicles (2.4 mg of protein/ml) were incubated in 32 mM ammonium acetate pH 5.4-6.25 and maintained at 37°C in a continuously stirred vessel. The inactivation was terminated by adding an equal volume of 0.1 M Tris-HCl, pH 7.4, 35 mM sucrose at 0°C. The final pH of the reaction mixture was 7.4. Control vesicles were incubated in 32 mM ammonium acetate pH 7.0. At this pH no inactivation of transport was apparent. Calcium transport activity (determined as described in Section 2.6) declined in a first order manner in the pH range 5.4-6.0 at 37°C (Figure 1a).

90% Inactivation of Ca^{2+} transport was obtained after 3 min at pH 5.4 and 5 min at pH 5.66 and at 37°C.

In the case of EGTA inactivation, SR vesicles (2.4 mg of protein/ml) were incubated in 0.1 M Tris-HCl, pH 7.4 and 10 mM EGTA at 37°C in a continuously stirred vessel for 5 min. The reaction was terminated by the addition of an equal volume of 0.1 M Tris-HCl, pH 7.85 and 10.2 mM $CaCl_2$. The final pH of the reaction mixture was 7.4. Control preparations were incubated in the presence of 10 mM EGTA and 10.2 mM $CaCl_2$ (free Ca^{2+} concentration was 100 μM). Similarly, EGTA inactivation of Ca^{2+} transport occurred in a first order manner (Figure 1b) with 10% transport activity remaining after 4 min incubation in EGTA at 37°C. Addition of 10.2 mM calcium during the inactivation process prevented further loss of transport activity. There was approx. 5% decline in calcium transport activity over 4 min on preincubation of vesicles at 37°C prior to assaying calcium transport (Figure 1b - control preparation).

In both cases the rate of inactivation was accelerated by using more dilute protein suspensions.

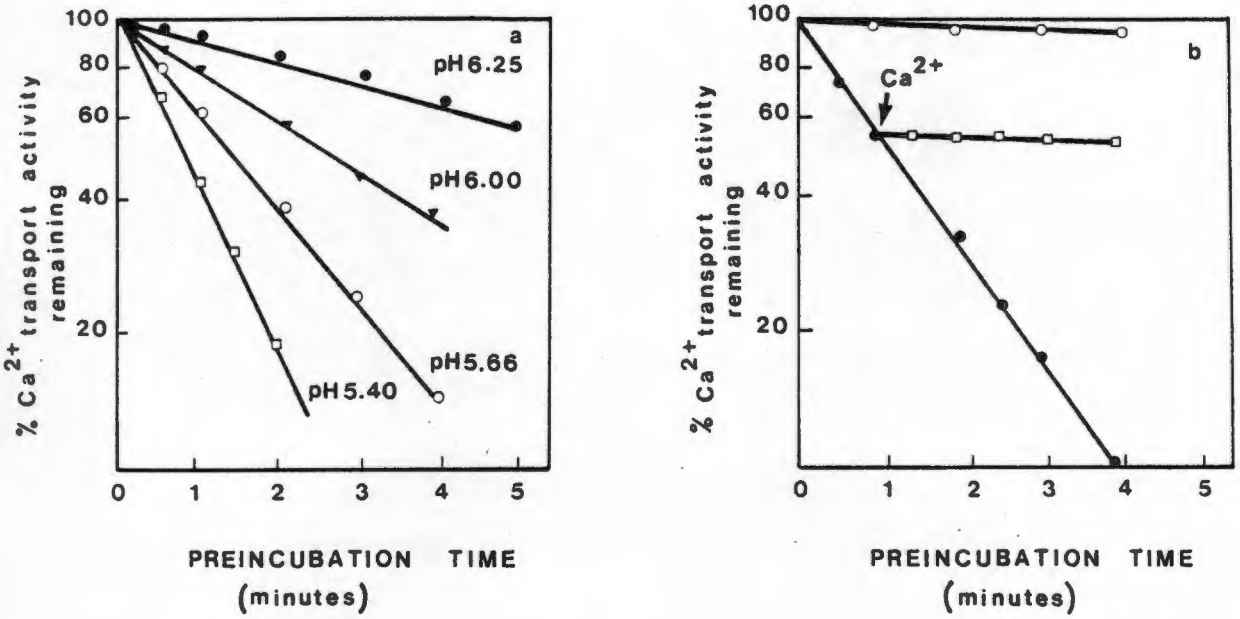


FIGURE 1 Acid-and EGTA-Inactivation of Calcium Transport by SR Vesicles

SR vesicles, 2.4 mg/ml, were preincubated in (a) 32 mM ammonium acetate pH 5.40 (□), pH 5.66 (○), pH 6.00 (▼), pH 6.25 (●) and at 37°C. Aliquots were removed at time indicated and added to an equal volume of 0.1/M Tris-HCl, pH 7.4, 32 mM sucrose at 0°C. The final pH of the reaction mixture was 7.4. The samples were assayed for calcium transport by the Millipore filtration technique (2.6). In (b) the vesicles incubated in 0.1 M Tris-HCl, pH 7.4 and 10 mM EGTA (●) at 37°C. At times indicated aliquots were removed and added to an equal volume of 0.1 M Tris-HCl, pH 7.85 and 10.2 mM $CaCl_2$ at 0°C. In a separate experiment, inactivation was initiated by the addition of 10 mM EGTA. Calcium at a final concentration of 10.2 mM was added after 1 min.(□). Aliquots were removed at times indicated and assayed for calcium transport activity (Section 2.6). In a control experiment, SR vesicles were incubated in the presence of 10 mM EGTA and 10.2 mM Ca Cl_2 at 37°C (○).

2.2 CHEMICAL MODIFICATION OF SR VESICLES

2.2.1 DTNB Modification of SR Vesicles

This method was essentially similar to that described by Murphy (1976).

SR vesicles (0.6 mg of protein/ml) which had previously been acid inactivated with respect to calcium transport (as described in Section 2.1.2) were modified with DTNB at 25°C. DTNB was added to a final concentration of 4 mM (a minimal 50-fold excess over the sulphydryl concentration to obtain pseudo-first-order rates). The absorbance vs time was recorded with a thermostatted Aminco DW-2 dual wavelength spectrophotometer at 412 nm-500nm, where the product, nitromercaptobenzoate absorbs maximially. For more rapid measurements, a Morrow-Chance stopped-flow apparatus was connected to the DW-2 to obtain rapid mixing of the DTNB and protein suspension. The number of moles of sulphydryls was calculated using $\epsilon_{412-500}$ (see below) and expressed per 1.5×10^5 g of protein, since the ATPase protein which has approx. 95% of the cysteines of SR (MacLennan *et al.*, 1971, 1972; Ikemoto *et al.*, 1974) has a molecular weight of 105 000 and constitutes about 70% of the total protein (Inesi and Scales, 1974). The data was analysed for distinguishable kinetic classes of sulphydryl groups by semilogarithmic plots and a reiterative curve fitting procedures described in Results (Section 3.1.2).

Determination of the value of $\epsilon_{412-500}$ was carried out by taking absorbance readings of the nitromercaptobenzoate

product of DTNB at 412 nm and 500 nm on a Zeiss PMQ II spectrophotometer against buffer containing 10μM DTNB. Excess β-mercaptoethanol was added until no further increase in absorbance occurred. The extinction coefficient ϵ_{412} was found to be 1.35×10^4 similar to the value of 1.36×10^4 previously reported (Ellman, 1959). The extinction coefficient $\epsilon_{412-500}$ was found to be 1.232×10^4 (Table 3). This value was used to calculate the number of moles of suphydryl groups in all stopped flow measurements where absorbance values were measured at A_{412} minus A_{500}.

TABLE 3 Calculation of $\epsilon_{412-500}$

Extinction Coefficient	Absorbance Reading
A_{412}	0.7865
A_{500}	0.074
$A_{412-500}$	0.7125

2.2.2 N-Ethylmaleimide Modification of SR Vesicles

This procedure is a modified method of Hasselbach and Seraydarian (1966). SR vesicles (1.0-3.0 mg of protein/ml) were modified at 25°C by addition of 1-2 mM NEM and 1-^{14}C-NEM (amount of radioactivity varied in all the experiments and is stated in the legends) (New England Nuclear). At timed intervals 0.25 ml aliquots were removed and the reaction was terminated by precipitation with 3.0 ml 10% (w/v)

trichloroacetic acid (TCA) at 0°C. Unbound NEM was removed by two washings with 3.0 ml 10% (w/v) TCA. The protein precipitate was dissolved in 0.5 ml "Soluene" (Packard Ltd), washed into a scintillation counting vial with 10.0 mls "Dimilume" (Packard Ltd) scintillation fluid and assayed for radioactivity in a Beckman LS-233 liquid scintillation counter. For determination of the total amount of radioactivity added, for use in the calculation of the specific activity of NEM, 25 µl aliquot was removed from the reaction mixture into a scintillation counting vial. The protein was solubilized with 0.5 mls "Soluene", 10.0 mls "Dimilume" was added and the radioactivity assayed.

2.3 STRUCTURAL STUDIES OF (Ca^{2+}, Mg^{2+}) -ATPASE

2.3.1 Trypsinization of SR Vesicles

SR vesicles, 1 mg/ml, in 1M sucrose and 10 mM Tris-HCl, pH 7.4 were incubated at 25°C. Trypsin crystalline from beef pancreas (BDH biochemicals - activity about 7500 BAEE units per mg) was added to a final concentration of 1000:1 and 200:1 protein to trypsin and allowed to react for 5 min and 15 min to obtain the single and double cleavage products, respectively. The reaction was terminated by the addition of soya bean trypsin inhibitor to a final ratio of inhibitor to trypsin of 2:1 by weight. The reaction mixture was centrifuged at 110 000 x g (50 000 r.p.m.) for 30 min in rotor No. 50 in a Beckman Spinco model L ultracentrifuge.

2.3.2 Purification of the (Ca^{2+}, Mg^{2+}) -ATPase of SR Vesicles and Peptide Mapping of the Purified ATPase

Preparation of the purified enzyme for peptide mapping was carried out by the following procedures:

(a) Removal of extrinsic proteins

This method was essentially similar to that described by MacLennan (1970). SR vesicles, 10 mg/ml, were suspended in a solution containing 0.25 M sucrose, and 10mM Tris-HCl, pH 8.0. The suspension was made 1 M by addition of solid KCl and then 10% (w/v) deoxycholate (DOC), pH 8.0, was added to

give a final concentration of 0.1 mg of DOC per mg of protein. The preparation was incubated at 0°C for 10 min and the suspension was centrifuged at 160 000 x g in a Beckman Spinco model L ultracentrifuge for 40 min. The slightly cloudy (opalescent) supernatant was removed by careful aspiration to ensure that the loosely packed pellet remained intact. The pellet was then resuspended in the original volume of sucrose-Tris buffer and centrifuged at 160 000 x g for 35 min. The washing step was repeated one more time after removal of the clear supernatant and resuspension of the pellet in the same buffer. After the second wash, the pellet was suspended in 1% (w/v) ammonium bicarbonate (NH_4HCO_3), pH 7.8.

(b) Extensive digestion of the protein, purification of the soluble peptide and peptide mapping

This method was a modification of the method described by Michalak and MacLennan (1980). Digestion was carried out on 20 mg of protein by addition of L-1-tosyl amido-2-phenyl-ethyl chloromethyl ketone (TPCK) trypsin (280 1U/mg, Worthington Biochemical Co., Freehold, N.J.) to a final concentration of 100:1 protein to trypsin. The digestion was allowed to proceed for 18 hr at 37°C in a continuously stirred vessel. The resulting mixture was centrifuged at 120 000 x g for 60 min in a Beckman Spinco model L ultracentrifuge in 1% NH_4HCO_3, pH 7.8. The resulting supernatant was freeze-dried and the concentrated digested protein was then passed through a Sephadex G-100 column (40 cm × 1.5 cm) equilibrated with 1% NH_4HCO_3, pH 7.8. The column was connected to a Beckman

Uvicord LKB Bromma, 2089 Uvicord 111, at a wavelength of 280 nm and the elution profile of the higher molecular weight fractions and the peptides were monitored. The higher molecular weight fractions eluted in the void volume of the column and the purified, digested ATPase fractions which eluted shortly afterwards were pooled, freeze-dried and suspended in pyridine/H_2O/acetic acid (10/89.7/0.3) buffer, pH 6.5. The sample (10µl) was spotted on cellulose thin layer chromatography plates (Eastman Chromotogram Sheet, Eastman Kodak Company) and electrophoresis was carried out in pyridine/H_2O/acetic acid buffer, pH 6.5 at 400 V/plate for 40 min on a high voltage electrophoresis apparatus (Pherograph Original-Frankfurt, type "Mini" 65). The plates were dried overnight at room temperature and chromotography in the second dimension was performed for 3 hr in butanol/pyridine/acetic acid/H_2O (65:50:10:40). The plates were dried overnight and sprayed with 0.1% (w/v) ninhydrin in acetic acid/acetone (20:80) buffer to visualize the peptide spots. Radioactive areas on the plates were detected by scanning the chromatography plate using a gas flow proportional counter fitted with a plotter (Dünschict-Scanner II, Berthoid LB 2723).

2.4 ANALYTICAL TECHNIQUES

2.4.1 Sodium Dodecyl Sulphate Polyacrylamide Disc Gel Electrophoresis

The method was essentially similar to that described by Weber and Osborn (1969).

Protein (0.5-1.0 mg/ml) in 10 mM Tris-HCl, pH 7.4, was solubilized in 1% sodium dodecyl sulphate (SDS; BDH, Poole, England) and 0.5% β-mercaptoethanol. Electrophoresis was carried out in glass tubes (12 × 0.65 cm). The tubes were rinsed in distilled water, then in 'Photo-Flo' (Eastman Kodak, New York) solution (1:200 dilution) and oven dried at 110°C before use.

The gels were made as follows:
Gel buffer contained 0.029 M $NaH_2PO_4 \cdot H_2O$, 0.072 M $Na_2HPO_4 \cdot 2H_2O$ and 0.2% SDS. 10% acrylamide solution was made by dissolving 22.2 g of acrylamide and 0.6 g of methylenebisacrylamide in water to a final volume of 100 mls. Insoluble material was removed by filtration through filter paper (Whatman No. 1). This solution was stored in the dark at 4°C. 15 ml of gel buffer was deaerated and then 13.5 ml of 10% acrylamide solution was added. After further deaeration, 1.5 ml of freshly prepared ammonium persulphate solution (15 mg/ml) and 0.045 ml of N,N,N', N'-tetramethylethylenediamine were added. The solutions were mixed and immediately pipetted into the glass tubes to within 1.5 cm from the top of each tube. Before the gel hardened a few drops of water was layered on

top of the gel solution. Gelling occurred after about 30 min when an interface could be seen indicating that the gel had solidified. The water layer was poured off just before use and the tubes were placed in a Bio-Rad Model 150 gel electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif., 94804). The samples were placed on the surface of the gel and the tube filled to the top with gel buffer which had been diluted 1:1 with distilled water. Each sample contained tracking dye (3 µl, 0.05% Bromophenol blue in water), glycerol (1 drop), β-mercaptoethanol (5 µl), 10mM Tris-HCl, pH 7.4 (50µl) and protein solutions (10-15 µl) containing 10-50 µg of protein. The two compartments of the electrophoresis apparatus was filled with gel buffer, diluted 1:1 with water. Electrophoresis was performed at a constant current (Spinco Duostat) of 10 ma per tube with the positive electrode in the lower chamber. At this current density, the voltage across the electrodes was approx. 50 volts. The marker dye moved approximately ⅞ of the total distance through the gel in approx. 5 hours. The length of the gel and the distance moved by the dye were measured. The gels were expelled from the tubes by introducing water from a syringe, placed between the gel and glass wall and applying pressure on one end by the use of a pipette bulb. The gels were placed in small tubes and stained with a Coomassie brilliant blue solution prepared by dissolving 1.25 g of Coomassie brilliant blue in a mixture of 454 ml 50% ethanol and 46 ml glacial acetic acid. The gels were stained in approx. 7 hours

at room temperature. The gels were destained by first soaking them in a destaining solution (75 ml acetic acid, 50 ml ethanol and 875 ml water) for 30 min and then electrophoretically destaining them for 2 hours in a Shandon Transverse Disc Destainer (60 ma per gel) with destaining solution in each reservoir. The gels were stored in 7.5% acetic acid. The length of the gel after destaining and the positions of the blue stained protein bands were measured. The mobilities were calculated from the formula:

$$\text{Mobility} = \frac{\text{Distance of protein migration}}{\text{Length of gel after destaining}} \times \frac{\text{Length of gel before destaining}}{\text{Distance of dye migration}}$$

A semilogarithmic plot of molecular weight against mobility was used to determine the molecular weights of the membrane proteins, after plotting the mobilities of the prepared standard solution (Pharmacia Fine Chemicals - low molecular weight markers) against their known molecular weights (Figure 2).

Absorption profiles of the stained gels were determined at 578 nm with a Vitatron densitometer TLD 100 using a slit width of 2.5 × 0.25 mm.

Assaying of Radioacitivity

The gels were photographed and then sliced into 1 mm slices, after freezing in dry ice, using a Mickel Gel Slicer. Each slice was placed in a scintillation counting vial and 1.0 ml "Soluene" (Packard Ltd.) was added to each vial. The vials were heated at 50°C for 3 hours after which time the slices decolourized and all the protein and radioactivity

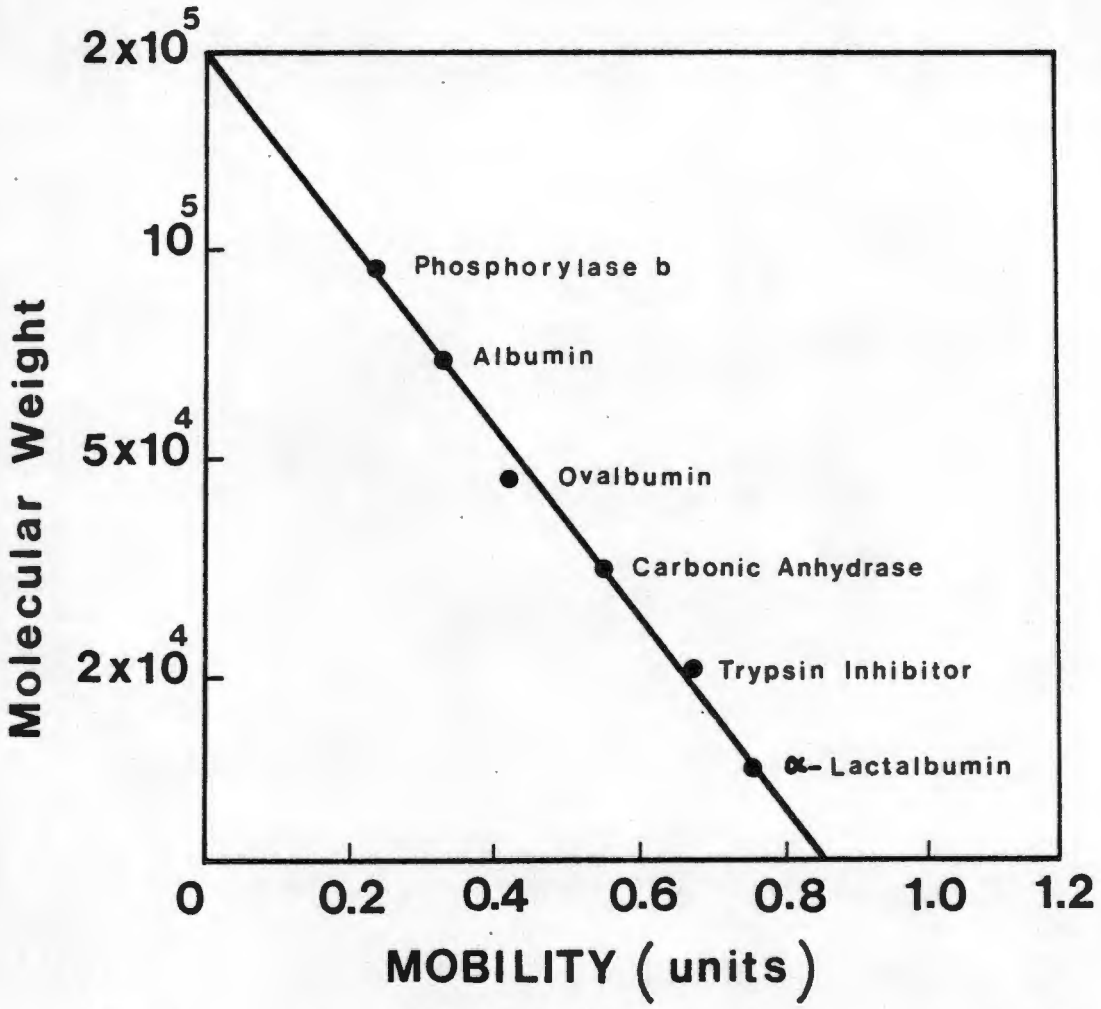


FIGURE 2 Standard Curve for the Determination of Molecular Weights of Proteins

Pharmacia Low Molecular Weight Electrophoresis Calibration Kit containing six proteins of approximately 100 µg of each protein and sucrose was dissolved in 100 µl of gel buffer containing 2.5% SDS and 5% β-mercaptoethanol by gentle swirling. The mixture was then heated at 100°C for 5-10 mins. Tracking dye was added and approximately 20 µg of each protein was applied to a 10% polyacrylamide gel containing 0.1% SDS. Electrophoresis was performed at a constant current of 10 ma per tube and was continued until the marker dye had moved $^{7}/_{8}$ of the distance down the tube. The protein band was stained with Coomassie blue and destained as described in "Methods". Mobility was calculated as follows:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before destaining}}{\text{distance of dye migration}}$$

The mobility of each standard protein shown was plotted against the logarithmic value of their known molecular weight. The molecular weights of proteins were determined from the above standard curve after calculating their mobilities.

eluted from the gel slice. After cooling 10 mls of "Insta-fluor" (Packard Ltd.) was added and the radioactivity in each gel was assayed for in a Beckman LS-233 liquid scintillation counter. The amount of radioactivity in each gel slice was plotted above the densitometric scan of the gel.

2.4.2 Sodium Dodecyl Sulphate Polyacrylamide Slab Gradient Electrophoresis

The method was a modified method of Laemmli (1970).

The following buffers were made for use in preparing the gradient gel:

Buffer A consisted of 1 M Tris-HCl, pH 8.8 and 30% glycerol; buffer B,1 M Tris-HCl, pH 8.8 and 7.5% glycerol; buffer C, 0.125 M Tris-HCl, pH 6.8 and tank buffer, glycine (60 g), buffer A (133 ml), 10% SDS (40 ml) made up to 2 li with water. The gradient gel for a 5% - 20% gel was prepared by making up the following mixture: 20% solution consisted of 30% cyanogum (16 ml), buffer A (8 ml) and 10% SDS (0.3 ml); 5% solution contained 30% cyanogum (4 ml), buffer B (8 ml), 10% SDS (0.3 ml) and 12 ml water. N, N, N′, N′-tetramethylethylenediamine (TEMED) (0.006 ml) and 0.2 ml of ammonium persulphate (50 mg/ml) were added to both solutions to assist in polymerization of the gel. The solutions were immediately added to a gradient former and the 20-5% gradient gel was prepared by running the solutions between two flat glass plates 18 cm × 26 cm and 18 cm × 23 cm, respectively, separated by spacers 1.5 mm in width and sealed with 2% agarose.

Water was carefully layered on top of the gel by means of a pasteur pipette and the gel was allowed to harden. After 30-40 mins gelling was complete and an interface could be seen between the solid gel and the water. The water was poured off and stacking gel, consisting of 30% cyanogum (1.2 ml), buffer C (8.6 ml), 10% SDS (0.3 ml), TEMED (0.006 ml) and ammonium persulphate (0.08ml 50 mg/ml), was placed on top of the gradient gel. Before gelling a perspex comb containing compartments for inserting the protein samples were placed in the stacking gel. The stacking gel hardened after 30-40 min. The comb and the bottom spacer were removed and the gel placed in a gel tank containing tank buffer,diluted 1:1 with water,placed in the upper and lower reservoirs. The protein samples were prepared as described for the disc gels and these as well as the standards were carefully placed in the defined spaces, made by the comb, in the stacking gel, using a Hamilton syringe. The electrophoresis was performed using a Spinco Duostat and the voltage across the electrodes was approx. 90 volts. After about 16 hours the bromophenol blue marker reached within 2 cm from the bottom of the gel. The gel was stained for 3 hours at room temperature in Coomassie brilliant blue solution and destained for approx. 7 hours in destaining solution. Both solutions were identical to those used for the disc gels. After removing all the ethanol by soaking the gel in 7.5% glacial acetic acid, the gel was dried using a Slab Gel Dryer (Hoefer Scientific Instruments) by placing the gel between Whatman 3M chromotography paper and photra graphic arts

film. The upper film layer was removed and the gel was scanned for radioactive peptides and proteins using a gas flow proportional counter (Dünnschict -Scanner II, Berthoid LB 2723). The molecular weights of protein bands were determined using a calibration curve which was plotted as described for the disc gels.

2.4.3 Determination of Protein Concentration

Protein concentrations were determined by the Biuret method of Gornall et al. (1949) and Lowry et al. (1951). The Biuret method was preferred at higher protein concentrations (approx. 10 mg protein/ml) whereas the Lowry method was used for lower protein concentrations.

(a) Biuret Method

The samples, 0.10 ml, containing 1.0-2.5 mg protein were mixed with 10% sodium deoxycholate (DOC) solution (0.10 ml). The solution was allowed to stand for 5 min for solubilization to take place and then Biuret reagent (5 ml) was added with mixing. After 30 min the absorbance was measured at 540 nm. Standards of bovine serum albumin (Sigma, St Louis, U.S.A.) were determined with each batch of samples and the calibration curve (range 10-50 mg/ml) was used to calculate the unknown protein concentration. Duplicate readings of all the protein determinations were performed.

(b) Lowry Method

The protein samples, 0.005-0.02 ml, containing 15-40µg protein were mixed with 10% DOC (0.05 ml) and the volume was

made up to 0.2 ml with water. Alkaline copper solution (1 ml) (containing 0.015% cupric sulphate, 0.05% sodium tartrate 2% sodium carbonate and 0.1M sodium hydroxide) was added and the mixture allowed to stand for 10 min. Folin-Ciocalteau reagent (0.10 ml) was added with vigorous mixing and the colour allowed to develop for 30 min. Protein concentrations were determined from changes in the optical density at 750 nm. Standards of 12.5, 25,50, 75 µg bovine serum albumin (Sigma, St. Louis, U.S.A.) were determined with each batch of samples. The unknown protein concentrations were calculated from the standard curve. The protein determinations were performed in triplicate and the average taken. Tris-buffer and sucrose appeared to enhance the colour development and these solutions were included into the standards when they were present in the samples.

2.5 (Mg^{2+}) - ATPase (EC 3.6.1.4) and (Ca^{2+}, Mg^{2+})-ATPase (EC 3.6.1.5) Activities

The ATPase activities were determined by the NADH-coupled method as described by Horgan et al. (1972). The hydrolysis of ATP is coupled to the oxidation of reduced nicotinamide-adenine dinucleotide (NADH) by including in the medium phosphoenolpyruvate (PEP), pyruvate kinase (PK) and lactate dehydrogenase (LDH). The assay was carried out at 30°C in 1 cm curvettes in the thermostatted cell compartment of an Aminco-DW2 dual-wavelength spectrophotometer, at 340 nm. The reaction mixture, 2.5 ml, contained 20mM histidine, pH 6.85, 50mM KCL, 5mM $MgCl_2$, 0.5 mM EGTA, 2.5mM PEP, 0.1mM $NADH_2$, 8 units/ml PK, 8 units/ml LDH and approx. 0.01 mg/ml of SR protein. The reaction was initiated by the addition of 1.0 mM ATP and the change in absorbance per min (ΔA/min) of the basal-ATPase activity ((Mg^{2+})-ATPase) was recorded. Ca^{2+}-dependent ATPase activity was initiated by the addition of 0.5mM $CaCl_2$ (free Ca^{2+} ion concentration = 11.2μM*) and the ΔA/min for the (Ca^{2+}, Mg^{2+}) -ATPase was recorded. The activity (μ mol Pi released/min/mg SR protein) was calculated using a molar extinction coefficient of 6.22 for NADH. Extra-ATPase activity was calculated by subtracting the (Mg^{2+}) -ATPase activity from the (Ca^{2+}, Mg^{2+}) -ATPase activity.

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*The concentration of free calcium ions in Ca^{2+}/EGTA buffer was calculated assuming dissociation constants (K_D) of 4×10^{-6} M^{-1}, 2.6×10^{-7} M^{-1} and 4.2×10^{-8} M^{-1} at pH 6.8, 7.0 and 7.4, respectively (Schwarzenbach, 1957).

2.6 ATP Dependent Calcium Uptake by Sarcoplasmic Reticulum Vesicles

Calcium transport by SR vesicles was determined by the following two procedures:

(a) Millipore filtration technique

This method was essentially similar to that previously described by Martonosi and Ferretos (1964). The amount of radioactive calcium transported into the SR vesicles was measured by the radioactivity remaining on a Millipore filter. Oxalate, a freely permeable anion, was included into the reaction mixture to ensure that the transported Ca^{2+} is precipitated within the vesicular lumen as insoluble calcium oxalate. The ATP dependent calcium uptake activity was measured at 25°C in an assay mixture containing 20 mM histidine, pH 6.85, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $^{45}CaCl_2$ (3 000 cpm/nmol; free Ca^{2+} ion concentration = 11.2 μM), 5 mM K^{+}-oxalate and 10-50 μg SR protein. The reaction was initiated by the addition of ATP and terminated at various time intervals by filtering a 0.20 ml aliquot through a Millipore Filter (type HA, 0.45 μ pore size) and washing with 15 ml water. As soon as washing commenced, the reaction was considered to be complete. The dried filter discs were placed in scintillation counting vials and 10 ml of Instagel (Packard Ltd.) was added and the radioactivity was assayed in a Beckman LS-233 liquid scintillation counter. The amount of calcium transported per mg protein, calculated from the radioactivity remaining on the filter, was plotted as a function

of time (Figure 3). The initial rate of uptake was linear for at least 1 minute and the reaction was complete after 2 min. The calcium uptake velocity (nmol Ca^{2+}/min/mg) was calculated from the slope of the curve. Controls containing no ATP were assayed for each preparation and the values which were < 1% were subtracted from the test values (Figure 3).

(b) Calcium specific electrode method

ATP dependent Ca^{2+} transport by SR vesicles was also measured by a Ca^{2+}-selective electrode (Radiometer type F2112-Ca) with a KCl reference electrode (Radiometer type K801). The calcium and reference electrodes were immersed in a jacketed vessel (capacity 10 mls) and the temperature was thermostatically controlled at 25°C. The electrodes were connected to a pH meter (Radiometer type PHM 62), and the output from this connected to a Hewlett-Packard 7004B recorder. The incubation mixture, 4.0 ml, contained 20 mM histidine, pH 6.85, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM K^+-oxalate and 0.02-1.00 mg/ ml SR protein. The reaction was initiated by the addition of ATP and recorded until complete. The electrode was previously calibrated with 25 µM, 50 µM, 75 µM and 100 µM $CaCl_2$ solutions which had been standardized by atomic absorption spectrophotometry with a Varian Techtron Atomic Absorption Spectrophotometer type AA. Absorption of emission from the Ca^{2+} lamp was determined at 422.7 nm in a nitrous oxide-acetylene flame. The ATP dependent calcium uptake was calculated from the slope of the curve and expressed as nmol Ca^{2+} transported/min/mg protein.

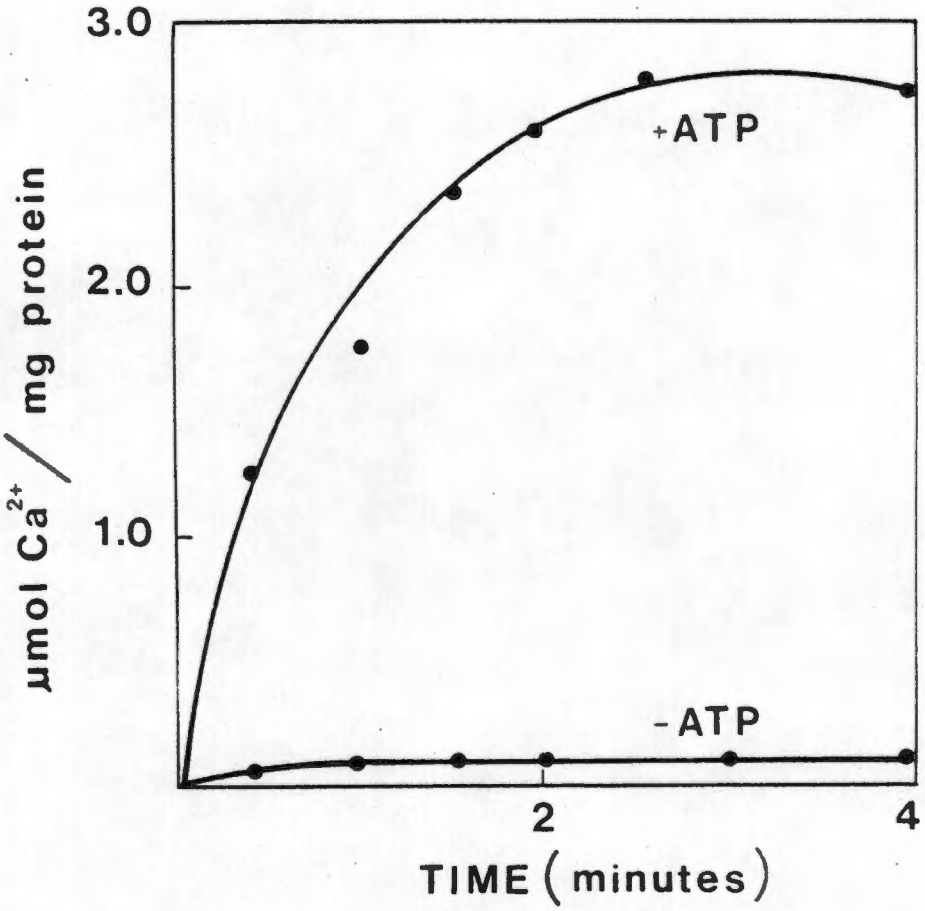


FIGURE 3 Kinetics of ATP-Dependent Ca^{2+} Transport by SR Vesicles

Calcium uptake was measured at 25°C in the standard assay mixture ("Methods" - 2.6) containing 10 µg SR protein/ml. The reaction was initiated by the addition of ATP (5 mM) and terminated at the times indicated by filtering through a Millipore filter. After washing, the $^{45}Ca^{2+}$ remaining on the filter was assayed in a scintillation counter.

3.0 RESULTS

3.1 ACID- AND EGTA-UNCOUPLING OF CALCIUM TRANSPORT FROM ATPase ACTIVITY IN SR VESICLES

Incubation of SR vesicles under mild acid conditions or in the presence of millimolar concentration of EGTA caused an inhibition of calcium transport and an enhancement in ATPase activity by approximately 70-75% (Figure 4). These phenomena have previously been described by Berman et al. (1977) and McIntosh and Berman (1978). These "uncoupled" preparations were used in the following experimental sections. Inactivation of calcium transport was carried out to the extent of 80-90% in most of these studies unless otherwise stated. The uncoupling reaction has previously shown to be irreversible (Berman et al., 1977; McIntosh and Berman, 1978). Following inactivation of calcium transport with EGTA, excess $CaCl_2$ was added such that the final concentration of free calcium was approximately 100 μM. This level of calcium has been shown to stabilize the calcium transport system of SR (McIntosh and Berman, 1978).

3.1.1 The Effects of Uncoupling of Calcium Transport of SR Vesicles on Thiol Group Reactivities

3.1.2 Kinetics of the DTNB Reaction of Acid-Inactivated SR Vesicles

Sarcoplasmic reticulum vesicles were prepared by a modified method of Eletr and Inesi (1972) as described in "Materials and Methods".

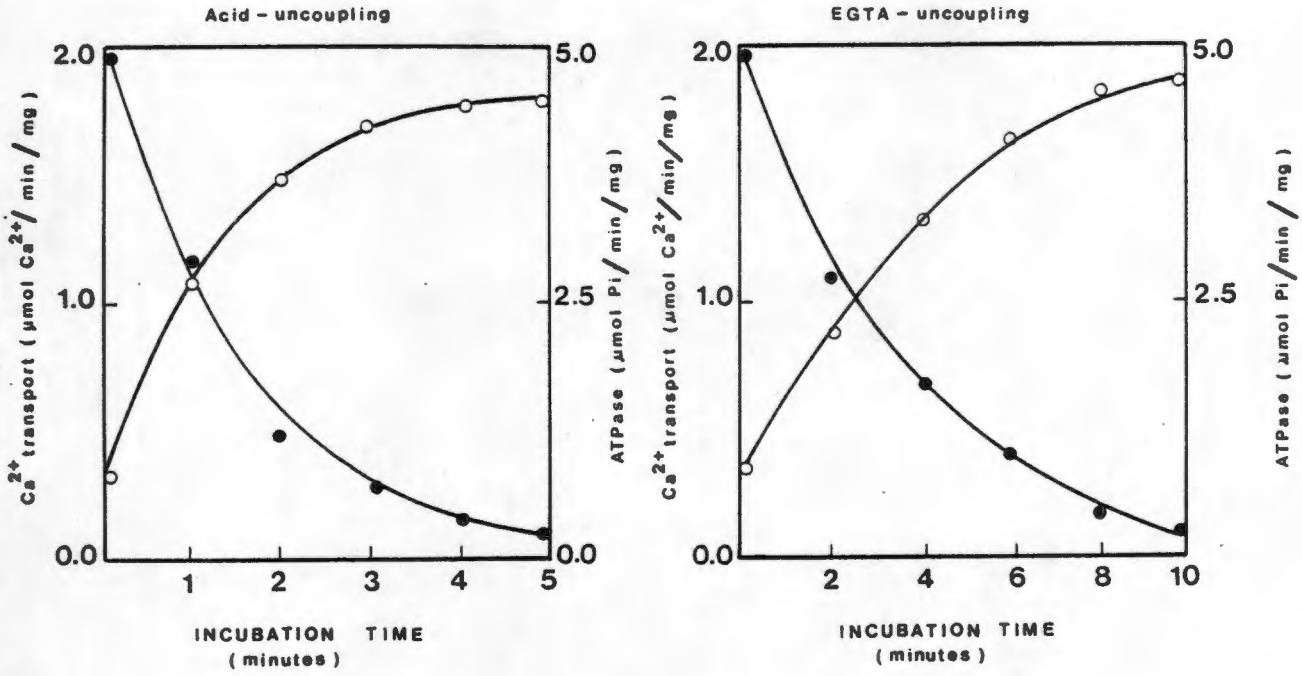


FIGURE 4 Uncoupling of Calcium Transport from (Ca^{2+}, Mg^{2+}) -ATPase Activity in Acid-and EGTA-Treated SR Vesicles

SR vesicles, 2.4 mg/ml, were incubated in 32 mM ammonium acetate pH 5.45 - 5.65 and maintained at 37°C in a continuously stirred vessel. The inactivation was terminated by adding to an equal volume of 0.1M Tris-HCl,pH 7.4 and 35 mM sucrose at 0°C.

In the case of EGTA inactivation, SR vesicles, 2.4 mg/ml, were incubated in 0.1M Tris-HCl pH 7.4 and 10mM EGTA at 37°C in a continuously stirred vessel. The reaction was terminated by the addition of an equal volume of 0.1M Tris-HCl,pH 7.85, 10.2 mM $CaCl_2$. The final pH of the reaction mixture was 7.4.

In both experiments aliquots were removed at the times indicated and assayed for Ca^{2+} transport activity, using the Millipore filtration technique (●) (2.6) and Ca^{2+} - stimulated ATPase activity was measured spectropholometically using an NADH-coupled method (O) (2.5).

The kinetic reactivity of the thiol groups of isolated SR vesicles was determined spectrophotometrically using DTNB in a stopped-flow apparatus. This reaction was essentially similar to that previously described by Murphy (1976), who characterized 3 kinetic classes of reactive thiol groups using a manual mixing method. However, we found by using stopped-flow analysis an additional fast reaction which was complete within 5-10 seconds of mixing (Figure 5). In the case of the manual mixing technique, the fast reaction was over in the order of minutes and thus the very fast reacting groups could not be identified.

Preincubation of SR vesicles at pH 5.5 and 37°C results in rapid inactivation of calcium transport without inhibition of (Ca^{2+}, Mg^{2+})-ATPase activity (Berman et al., 1977). Following incubation at pH 5.5, the calcium transport declined in a first order manner (Figure 5 inset). Aliquots were removed at the time intervals indicated and the thiol reactivity was determined. The results were analysed by an iterative curve-fitting technique, in terms of three exponential functions, using initial estimates for parameters obtained by graphical curve stripping (Murphy, 1976), (Figure 6). The three sets of thiol groups corresponding to the three exponential functions are referred to as fast, moderately fast and slowly reacting thiol classes and are represented by the following equation:

$$SH_t = SH_1 e^{-k_1 t} + SH_2 e^{-k_2 t} + SH_3 e^{-k_3 t}$$

where

SH_t = number of reactive thiol groups at time t;
SH_1 = number of fast reacting thiol groups;
SH_2 = number of moderately fast reacting thiol groups;
SH_3 = number of slowly reacting thiol groups,

and k_1, k_2 and k_3 are pseudo-first-order rate constants of each kinetic class of -SH groups respectively. In addition to the above kinetic classes, a fourth unreactive thiol class was measured as the difference between these reactive thiol groups and the total number of reactive thiols, determined by DTNB following incubation in SDS (1%;w/v). The results are summarized in Table 4.

Acid inactivation caused an increase in the number of -SH groups forming part of the fast and moderately fast kinetic classes, with a decrease in total number of reactive thiol groups. Total reactive thiol groups increased from approx. 15-16 to 18 during the first 15 seconds of acid treatment, mainly due to an increase in the slowly reacting groups, and declined slowly thereafter as a result of a decrease in slowly reacting -SH groups. Near complete inactivation of calcium transport resulted in the appearance of approx. 1 fast reacting thiol and 1-2 intermediate groups with a decrease in slowly reacting groups.

In summary, acid inactivation of calcium transport of SR membranes was accompanied by the appearance of 2-3 fast reacting thiols and a decrease in slowly reacting thiol groups,

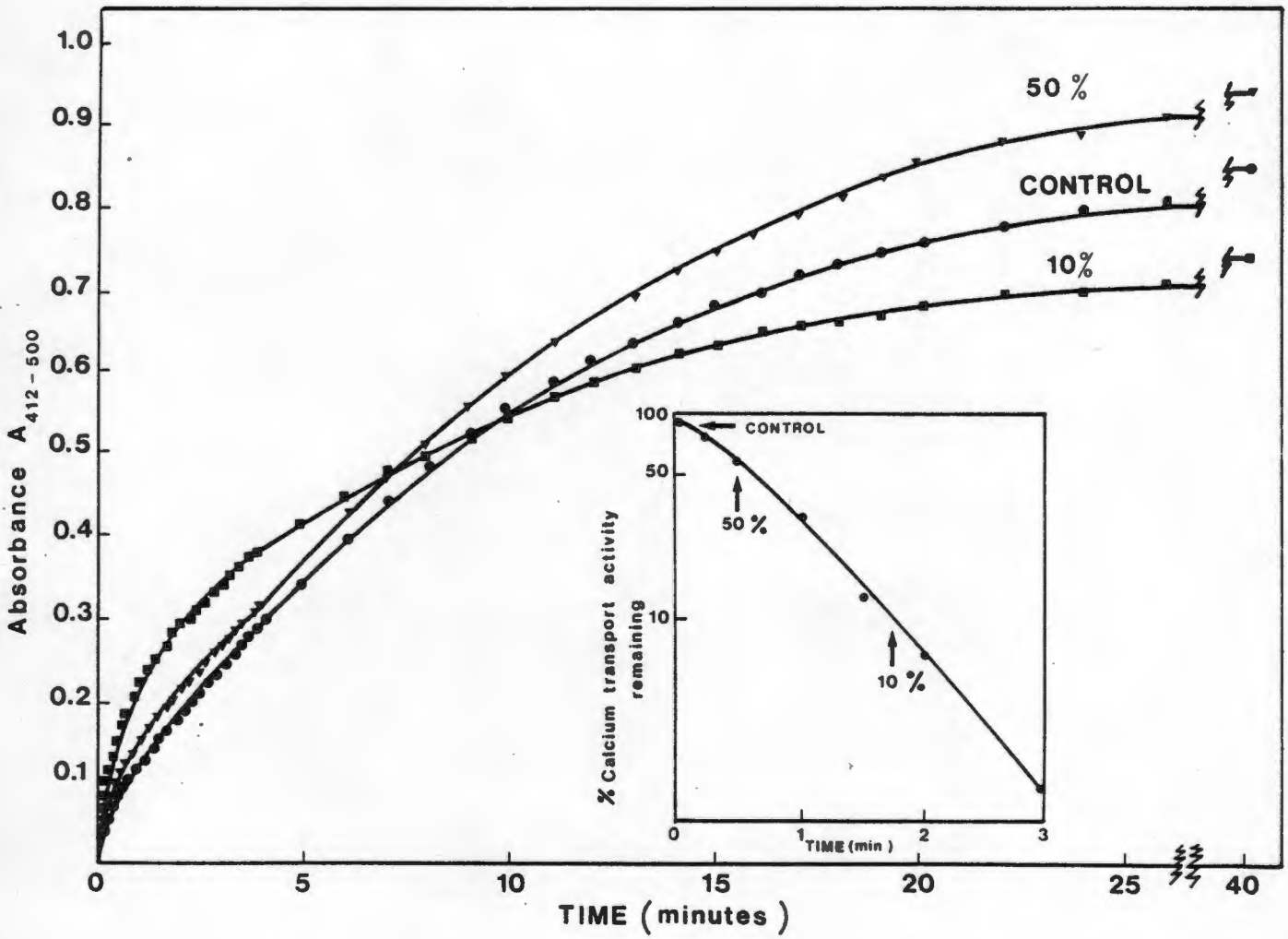


FIGURE 5 Kinetics of the DTNB Reaction of Acid-Inactivated SR Vesicles

SR vesicles, 0.6 mg/ml, were inactivated with acid in an ammonium acetate buffer composition at pH 5.5 as described in 'Methods'. At timed intervals during the inactivation (inset) aliquots were removed and the inactivation terminated. The samples were stored at 0°C and then assayed for -SH reactivity with the DTNB reaction by the stopped flow method as described in 'Methods'. Control preparations were incubated in ammonium acetate buffer at pH 7.0. The total -SH content was determined by incubation of SR vesicles, 0.6 mg/ml, in 10 mM Tris-HCl pH 7.4. SDS was added to a final concentration of 1% (w/v) and the vesicles were allowed to stand at 25°C for 10 mins. before the -SH group reactivity was estimated spectrophotometrically by the DTNB reaction (see 'Methods').

See Table 4 for the number of -SH groups in each kinetic class i.e. SH_1, SH_2, and SH_3 and their pseudo-first-order rate constants k_1, k_2 and k_3.

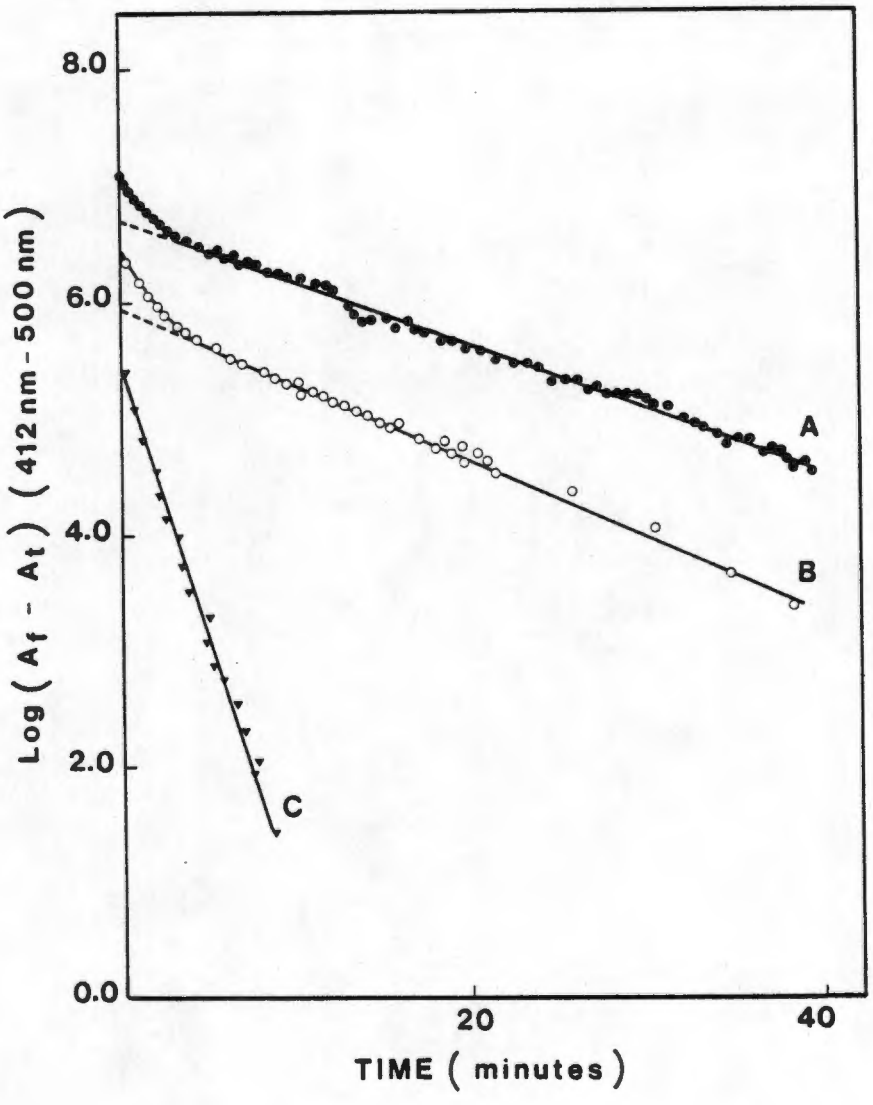


FIGURE 6 Graphical Curve Stripping

Data obtained from the kinetic reactivity of -SH groups of SR vesicles (reaction conditions as described in legend to Figure 5), determined spectrophotometrically using DTNB in a stopped flow apparatus, were analysed in terms of three-exponential functions. The logarithmic value of the difference in final absorbance, $A_{412-500}f$, and the absorbance at time t, $A_{412-500}t$, was plotted against time (Curve A). "Curve stripping" of the first and second slow semilogarithmic phases are shown by curves B and C, respectively.

TABLE 4 The Effect of Acid Inactivation on Calcium Transport and on the Amount and Kinetic Reactivity of -SH Groups of Isolated SR Vesicles

CONDITIONS	% Inactivation of Calcium Transport	k_1 $10^2 \times k(s^{-1})$	k_2 $10^2 \times k(s^{-1})$	k_3 $10^2 \times k(s^{-1})$	SH_1	SH_2	SH_3	Total Reactive -SH Groups	Unreactive -SH Groups
Control SR + 32mM NH_4Ac, pH 7.0 - incubate at 37°C for 10 min.	0	159.0	22.0	0.23	0.4	0.8	14.4	15.6	4.5
SR + 32mM NH_4Ac, pH 5.5 at 37°C for 0.25 min.	25	108.0	12.0	0.18	0.6	0.9	16.9	18.4	1.7
SR + 32mM NH_4Ac, pH 5.5 at 37°C for 0.5 min.	43	77.0	8.6	0.17	0.8	1.2	16.0	18.0	2.1
SR + 32mM NH_4Ac, pH 5.5 at 37°C for 1.0 min.	73	58.0	7.8	0.19	0.9	1.4	15.0	17.3	2.8
SR + 32mM NH_4Ac, pH 5.5 at 37°C for 1.5 min.	91	57.0	7.4	0.20	1.3	1.8	13.8	16.9	3.2
SR + 32mM NH_4Ac, pH 5.5 at 37°C for 2.0 min.	97	48.0	5.5	0.20	1.4	1.9	13.0	16.3	3.8
SR + 32mM NH_4Ac, pH 5.5 at 37°C for 3.0 min.	99	50.0	5.8	0.21	1.5	2.3	11.6	15.4	4.7
SR + SDS 1% (w/v) incubate at 25° 25°C for 10 min.	-	1353.0	28.0	0.18	9.3	6.5	4.3	20.1	0

resulting in a decrease in the total number of reactive -SH groups. This complex kinetic behaviour suggests that acid inactivation rendered a limited number of -SH groups more reactive to DTNB. The decrease in total number of thiol groups could have been due to autoxidation of these highly reactive -SH groups leading to disulphide bond formation. This possibility was therefore investigated using the -SH reagent 1-^{14}C-N- ethylmaleimide.

3.2 Thiol Reactivity of Acid - and EGTA-Treated SR Vesicles by Modification with N-Ethylmaleimide

3.2.1 Estimation of the Percentage Purity of 1-^{14}C-N-Ethylmaleimide

Previous reports (Hasselbach et al., 1966) have suggested that not all of the radioactive label in preparations of 1-^{14}C-NEM reacts with -SH groups. The percentage purity of 1-^{14}C-NEM was therefore estimated in order to correct for the amount of non-1-^{14}C-NEM radioactivity present. The radioactive purity of the preparation of 1-^{14}C-NEM was determined by reaction of trace amounts of the labelled compound with an excess of SR protein. The results are shown in Figure 7 where incorporation of the label of 1-^{14}C-NEM into the precipitable material was followed at timed intervals. The precipitated protein was rapidly labelled and by 10 mins. 80% of the total added radioactivity was found. Further slow incorporation occurred and by 120 min no further labelling was observed.

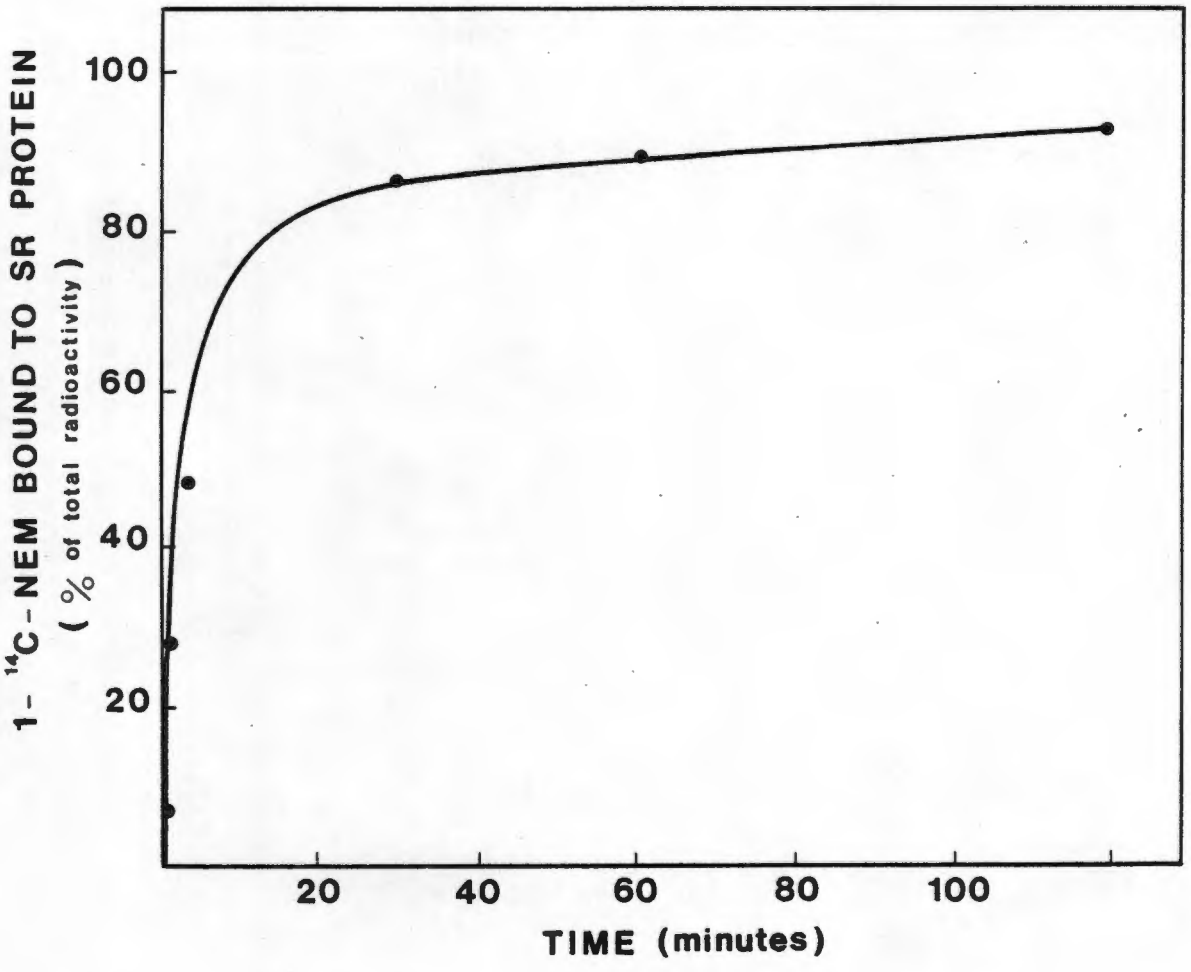


FIGURE 7 Determination of the Percentage Purity of 1-^{14}C-NEM

SR vesicles, 1mg/ml, were incubated at 25°C in 0.25 M sucrose, 10 mM imidazole pH 8.5. The reaction was initiated by the addition of 7.65 mmol 1-^{14}C-NEM (4.3×10^4 cpm/µmol). At timed intervals, 0.25 ml aliquots were removed and added to 3.0 mls 10%(w/v) TCA and stored on ice for 10 mins. The mixture was centrifuged at 3000 x g for 20 min. and the supernatant was decanted. The protein precipitate was resuspended in 3 mls 10%(w/v) TCA and recentrifuged. The washed precipitate was dissolved in 0.5 ml"Soluene"(Packard Ltd.), washed into a scintillation counting vial with 10 mls of"Dimilume"(Packard Ltd.) scintillation fluid and assayed for radioactivity (●——●). The results are expressed as a percentage of the total radioactivity present.

Since 93% of the label was bound at 60 min., 7% of the label was considered to be non-1-^{14}C-NEM.

This experiment showed that greater than 90% of the ^{14}C-label in the batch of labelled NEM used in the following studies was present as reactive 1-^{14}C-NEM. In all further calculations the labelled preparation was taken to be 93% pure. It would appear that 1-^{14}C-NEM is available in a higher state of purity than that employed previously (Hasselbach and Seraydarian, 1966).

3.2.2 Kinetics of the Reaction of 1-^{14}C-N-Ethylmaleimide with Control SR Vesicles and Following Acid- and EGTA-Treatment

Treatment of SR vesicles with EGTA or acid irreversibly inactivates transport and increases Ca^{2+} -ATPase activity, inset Figures 8b and 9 (Berman et al., 1977 and McIntosh and Berman, 1978). The effect of EGTA treatment on the covalent labelling of SR vesicles with ^{14}C-NEM at pH 7.4 is shown in Figure 8. Preincubation of the vesicles in the presence of millimolar concentrations of EGTA at 37°C and pH 7.4 caused an increase in the rates of labelling of the vesicles. This rate of labelling was enhanced when the reaction was performed at pH 8.0.

SR vesicles were EGTA inactivated at 37°C, pH 7.4, the inactivation was terminated by the addition of calcium. SR vesicles thus inactivated, to the extent of 90% of control SR transport activity, were reacted with 1-^{14}C-NEM, aliquots

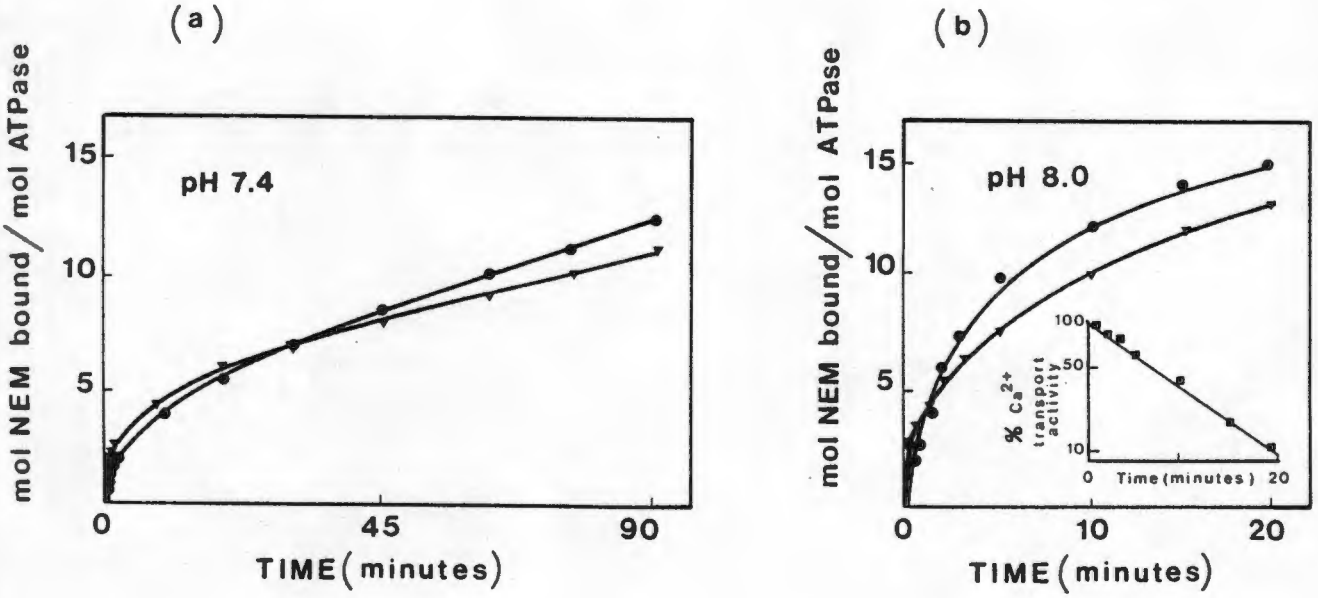


FIGURE 8 Kinetics of NEM-labelling with EGTA-Treated SR Vesicles

SR vesicles, 2.4 mg/ml, were incubated in 0.1 M Tris-HCl, pH 7.4 and 10.0 mM EGTA at 37°C for 18 min. (to obtain 90% in activation of Ca^{2+} transport activity), then brought to 25°C. The reaction was immediately initiated by the addition of an equal volume of 0.1M Tris-HCl, pH 8.0 for (a) and pH 8.75 for (b), 2mM NEM, 10.2mM Ca Cl_2 and 2.25 µM 1-^{14}C-NEM (200 cpm/nmole). The final pH of the reaction mixture was 7.4 and 8.0 for (a) and (b) respectively. At timed intervals 0.25 ml aliquots were removed and the protein was precipitated, centrifuged, solubilized and assayed for radioactivity as described in 'Method' (▼——▼). Control preparations consisted of SR vesicles, 2.4 mg/ml, 0.1 M Tris-HCl, pH 8.0, 10mM EGTA and 10.2 mM $CaCl_2$ incubated at 37°C for 18 min. The final pH of the reaction mixture was 7.4. The reaction was immediately brought to 25°C and initiated by the addition of an equal volume of 0.1 M Tris-HCl, pH 7.4 for (a) and pH 8.0 for (b), 2 mM NEM and 2.25 µM 1-^{14}C-NEM (200cpm/nmole). The final pH of the reaction mixture was 7.4 and 8.0 for (a) and (b) respectively. The remaining procedures were carried out as above (●——●).

were removed at the times indicated in Figure 8 and the enzyme-bound label quantitated.

EGTA-uncoupling of the (Ca^{2+}, Mg^{2+}) - ATPase resulted in an increase in the rate of binding of the radioactive NEM to the reactive -SH groups at pH 7.4 (Figure 8a). As NEM reacts faster at alkaline pH, the procedure was repeated at pH 8.0 (Figure 8b) and a similar trend was observed. At both pH values, 0.5 - 1.0 thiol groups/ATPase were increased in reactivity. However, the total number of reactive -SH groups decreased by approximately 1.0 - 2.0, when compared with control preparations (Figure 8).

The -SH group reactivity with NEM at pH 7.4, of SR vesicles which had been acid-inactivated by 90%, with respect to calcium transport activity by preincubation at pH 5.6 and 37°C, was similarly investigated (Figure 9). In agreement with EGTA-uncoupled vesicles, acid-uncoupling resulted in an increased rate of thiol binding and a decrease in total number of accessible -SH groups. However, the effect of acid treatment was more pronounced with respect to the greater increase in labelling of fast reacting thiol groups. Approximately 1.0 - 2.0 -SH groups were increased in reactivity and the total number decreased by approximately 1.0 - 2.0 thiol groups.

Several possible explanations for the decrease in total number of reactive -SH groups by acid- or EGTA-treatment were considered. (i) Previously reactive groups may have been rendered non-reactive. (ii) The increased reactivity of specific -SH groups may have rendered them susceptible to

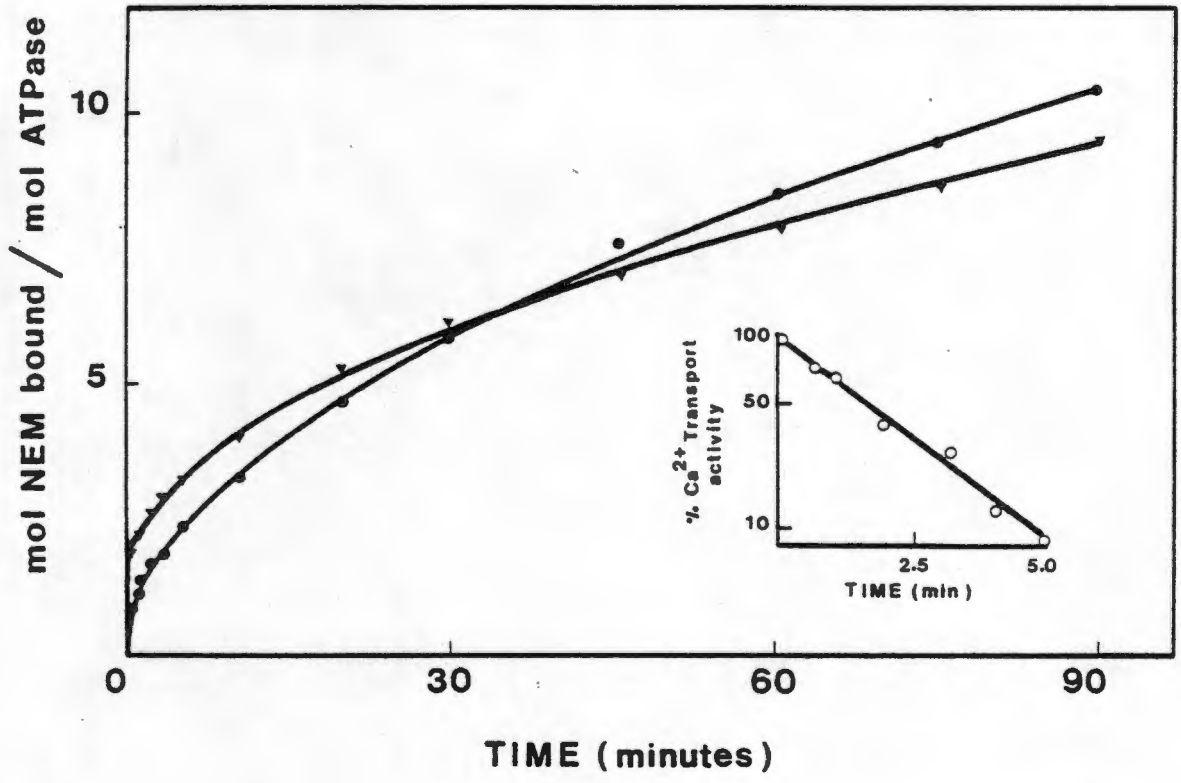


FIGURE 9 Kinetics of NEM-Labelling with Acid-Treated SR Vesicles

SR vesicles, 2.4 mg/ml, were incubated in 32 mM ammonium acetate, pH 5.6 at 37°C for 4 min. (to obtain 90% inactivation of calcium transport). Following equilibration to 25°C, the reaction was immediately initiated by addition of an equal volume of 0.1 M Tris-HCl, pH 7.4, 35 mM sucrose, 2mM NEM and 2.25 µM 1-^{14}C-NEM (200 cpm/nmole). The final pH of the reaction mixture was 7.4. Precipitation, centrifugation, solubilization and assaying of radioactivity of protein was carried out as described in 'Material and Methods' (▼—▼). Control preparations consisted of SR vesicles, 2.4 mg/ml, in 32 mM ammonium acetate, pH 7.0, incubated at 37°C for 4 min. The control vesicles were then treated as above (●—●).

autoxidation. The resultant disulphide bond formation may have occurred either as intramolecular disulphide or intermolecular cross-linkages between ATPase monomers or even possibly to other membrane proteins such as the glycoprotein, calsequestrin, or M55.

These possibilities were investigated in the following series of experiments.

3.3 Total Reactive Thiol Content of EGTA-Treated SR Vesicles

Total -SH reactivity of SR vesicles may be measured following treatment with SDS. The effect of inactivation of SR membranes with EGTA on the DTNB reaction in the presence of 1% (w/v) SDS is shown in Figure 10.

EGTA-inactivation of calcium transport, at 37°C (Figure 10, inset) resulted in a decrease in total number of reactive thiol groups. Prior to inactivation there were approximately 20 reactive thiol groups and after 90% inactivation of calcium transport with respect to control vesicles, only 18 - 19 - SH groups were reactive i.e. 1.0 - 2.0 thiol groups/ ATPase were no longer reactive in the presence of SDS (1%; w/v) (Figure 10). Incubation of SR vesicles in millimolar concentrations of EGTA at 0°C and 25°C, at pH 7.4, which did not inhibit calcium transport (Figure 10, inset), showed no decrease in total number of -SH groups. There were a total of 20 reactive thiol groups before and after incubation of EGTA with SR vesicles at 0°C and 25°C (Figure 10). Therefore

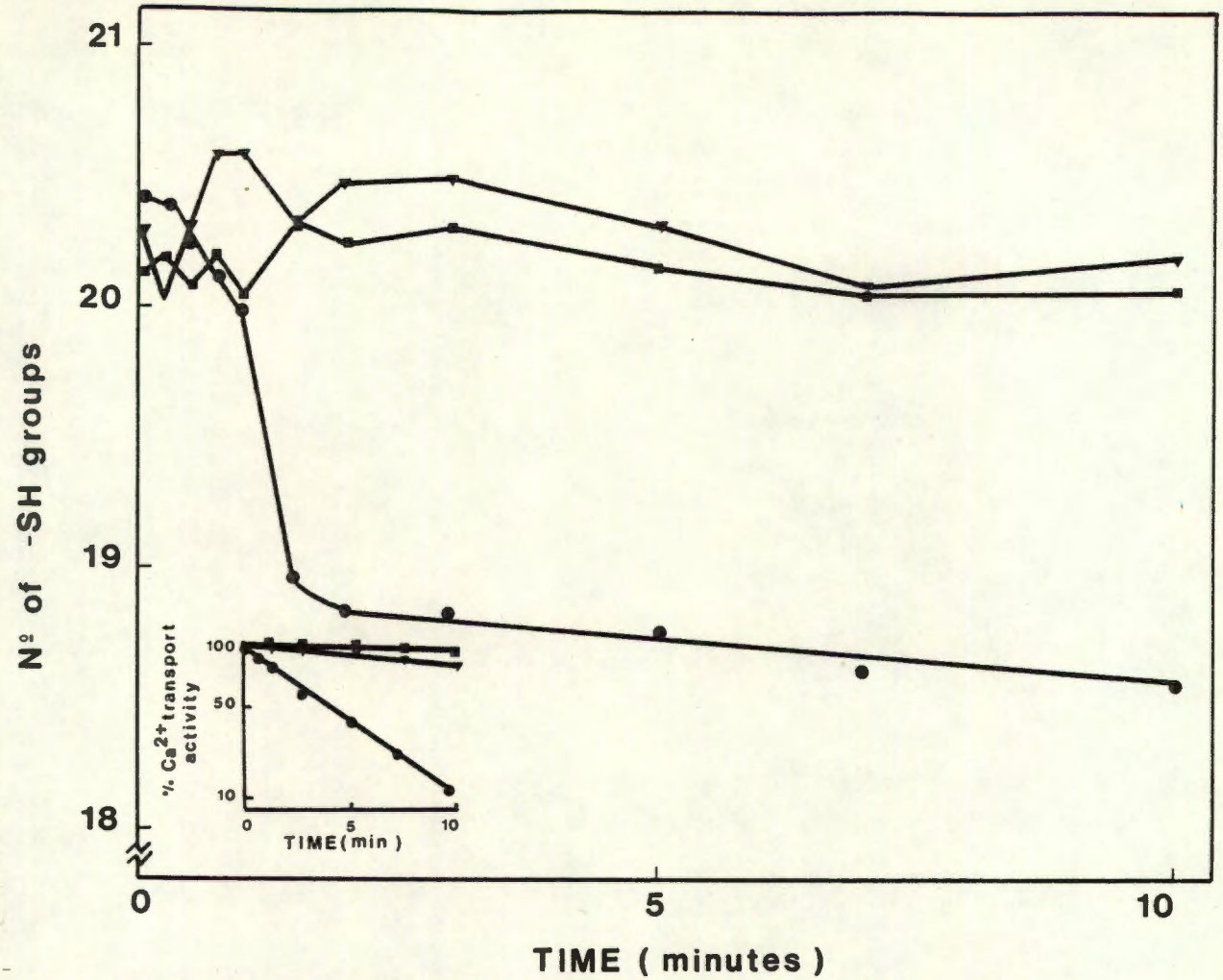


FIGURE 10 Total Reactive Thiol Content of EGTA-Treated SR Vesicles

SR vesicles, 2.4 mg/ml, were incubated in 0.1 M Tris-HCl, pH 7.4 and 10 mM EGTA at 37°C (●——●), 25°C (▼——▼) and 0°C (■——■). At timed intervals, 0.25 ml aliquots were removed and added to an equal volume of 0.1 M Tris-HCl, pH 8.0, 10.2 mM $CaCl_2$, at 25°C. The final pH of the reaction mixture was 7.4. SDS was added to a final concentration of 1% (w/v) and DTNB to a final concentration of 2 mM. The DTNB reaction was allowed to proceed for 40 min. and then the absorbance at 412 nm was measured.
Turbidity readings were taken by excluding DTNB from the reaction mixture (A_{412} (turbidity)) and the initial absorbance was measured in the absence of SR (A_{412} (initial)).
Corrections for turbidity and possible changes in turbidity were checked by including blanks containing SR or DTNB alone. Readings were zeroed against buffer and SDS (1%;w/v)(A_{412} (buffer)) and all readings were duplicated in balanced curvettes. The total number of -SH groups was calculated as follows:

$$-SH_{Total} = \left[(A_{412}(final) - A_{412}(initial)) - A_{412}(turbidity) - A_{412}(buffer)\right] \times \frac{MW_{SR}}{\varepsilon_{412}} \times \frac{1}{mg/ml}$$

where MW_{SR} = 150 000 daltons of protein, containing approx. 1 molecule of ATPase.

ϵ_{412} = extinction coefficient at 412 nm = 13.600 (Ellmann, 1959)

the decrease in total number of reactive thiols correlated with a decline in calcium transport activity.

The total number of reactive thiol groups obtained in our preparations, 20 -SH groups/ATPase, is in good agreement with the 20 cysteine residues, reported by Thorley-Lawson and Green (1977), on the unreduced (Ca^{2+}, Mg^{2+}) -ATPase. Since this protein constitutes 90% of SR vesicles preparations, (Figure 11), it is unlikely that any -SH groups are inaccessible in 1% (w/v) SDS.

The decreases in total number of thiol groups in the EGTA-uncoupled SR vesicles thus appears to be due to autoxidation, followed by disulphide bond formation and not due to internalization of the reactive -SH groups rendering them inaccessible to the thiol-specific reagent.

3.4 Quantitation of the Number of -SH Groups Involved in the Process of Uncoupling of Calcium Transport by EGTA Treatment

It is apparent from the data in Section 3.3, that the decrease in number of total reactive thiol groups, following treatment of SR vesicles with EGTA or acid, is not due to their inaccessibility to the -SH directed probes.

Two mechanisms appear to be responsible for the data shown in Figures 8 and 9. In the early stages of inactivation of transport, thiol group reactivity is increased, leading to disulphide bond formation. The latter process leads to an underestimate of the number of -SH groups whose reactivity is altered during inactivation of transport.

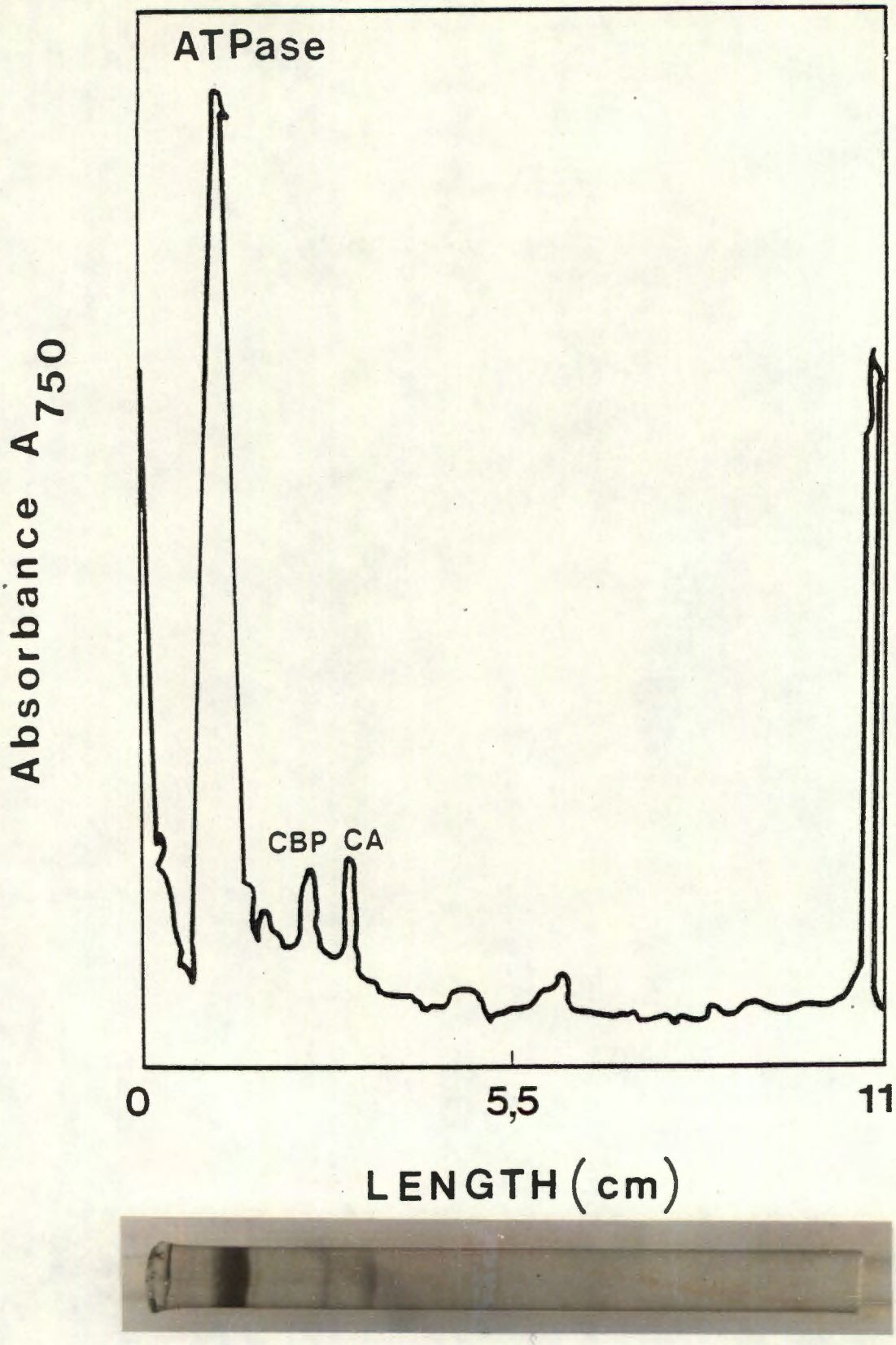


FIGURE 11 (Ca^{2+}, Mg^{2+}) -ATPase Content of SR Vesicle Preparations

SR vesicles, 0.6 mg protein, were solubilized in 1% (w/v) SDS and then dialysed for 16 hrs against 1% (w/v) SDS. Approx. 25 µg protein was layered on top of 10% polyacrylamide gels (Section 2.4.1) and electrophoresis was carried out with a current of 10 ma per gel for approx. 4 hrs. The protein bonds were stained in a solution containing 9.2% (v/v) acetic acid, 50% (v/v) methanol and 0.25% (w/v) Coomassie brilliant blue. Gels were destained in 7.5% (v/v) acetic acid and 5% (v/v) methanol electrophoretically for 4 hrs as described in "Methods". The absorbance profile at 578 nm of the stained gel is shown. CBP, Calcium-binding protein; CA, Calsequestrin.

This process was thus further investigated by inactivating the calcium transport in the presence of NEM which would react with exposed -SH groups immediately, thus preventing autoxidation. It has previously been shown that 0.5 - 2.0 -SH groups/ATPase, which react very rapidly with -SH reagents are not important or involved in the uncoupling procedure (Sections 3.1.2 and 3.2.2). In order to achieve greater selectivity an experiment was designed whereby these fast groups were initially 'activated' by incubation of SR vesicles for 2 mins. at 37°C with labelled NEM in the absence of EGTA. This allowed for easy distinction of the fast reactive groups which become exposed during EGTA-uncoupling. After 2 min. preincubation with NEM, millimolar concentrations of EGTA were added to initiate inactivation of calcium transport (Figure 12). This treatment resulted in approx. 1.0 added -SH group/ATPase being exposed in EGTA-treated vesicles compared with control, untreated vesicles. The rate constant for the increase in 1 -SH group was calculated to be 1.45 min^{-1}.

In summary, there appears to be approx. 1.0 -SH group/ATPase involved in the process of uncoupling. This is a fast reacting group and is readily autoxidized to form a disulphide bond.

For further procedures a pilot experiment was designed to determine the optimum time for blocking approx. 4 fast reacting thiol groups, which do not appear to be involved in the uncoupling procedure. It was found (Figure 13) that in the presence of 5 mol NEM/mol ATPase at 25°C, approx. 4 -SH groups reacted after 90 min. This time was therefore selected for further experiments.

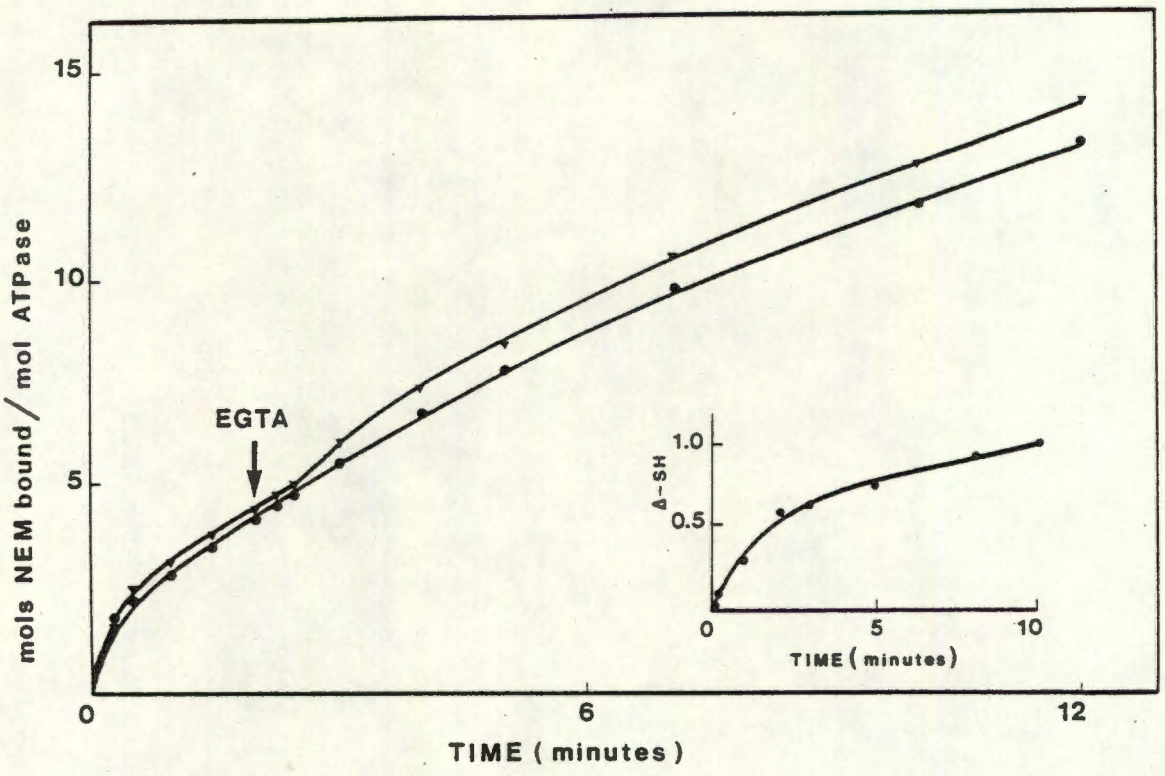


FIGURE 12 **Quantitation of the Number of -SH Groups Increased in Reactivity during Uncoupling of Calcium Transport by EGTA-Treatment**

SR vesicles 1.2 mg / ml, were incubated at 37°C in 0.1 M Tris-HCl, pH 7.4, 1 mM NEM and 3.5 µM 1-^{14}C-NEM (320 cpm/nmole). After 2 min incubation, the inactivation of calcium transport was initiated by addition of EGTA to a final concentration of 5 mM (▼—▼). No EGTA was added to control experiment (●—●). At times indicated 0.25 ml aliquots were removed and precipitation, centrifugation, solubilization and assaying of radioactivity was carried out as described in "Methods".

Inset:

Difference in the number of reactive -SH groups in EGTA-treated vesicles to control preparations estimated after the addition of EGTA (i.e. 2 min).
i.e. ΔSH = ΔSH (EGTA-treated vesicles - CONTROL vesicles) mol/mol ATPase.

NOTE: In the first two minutes the curves for control and EGTA treated vesicles are superimposable but have been separated for clarity.

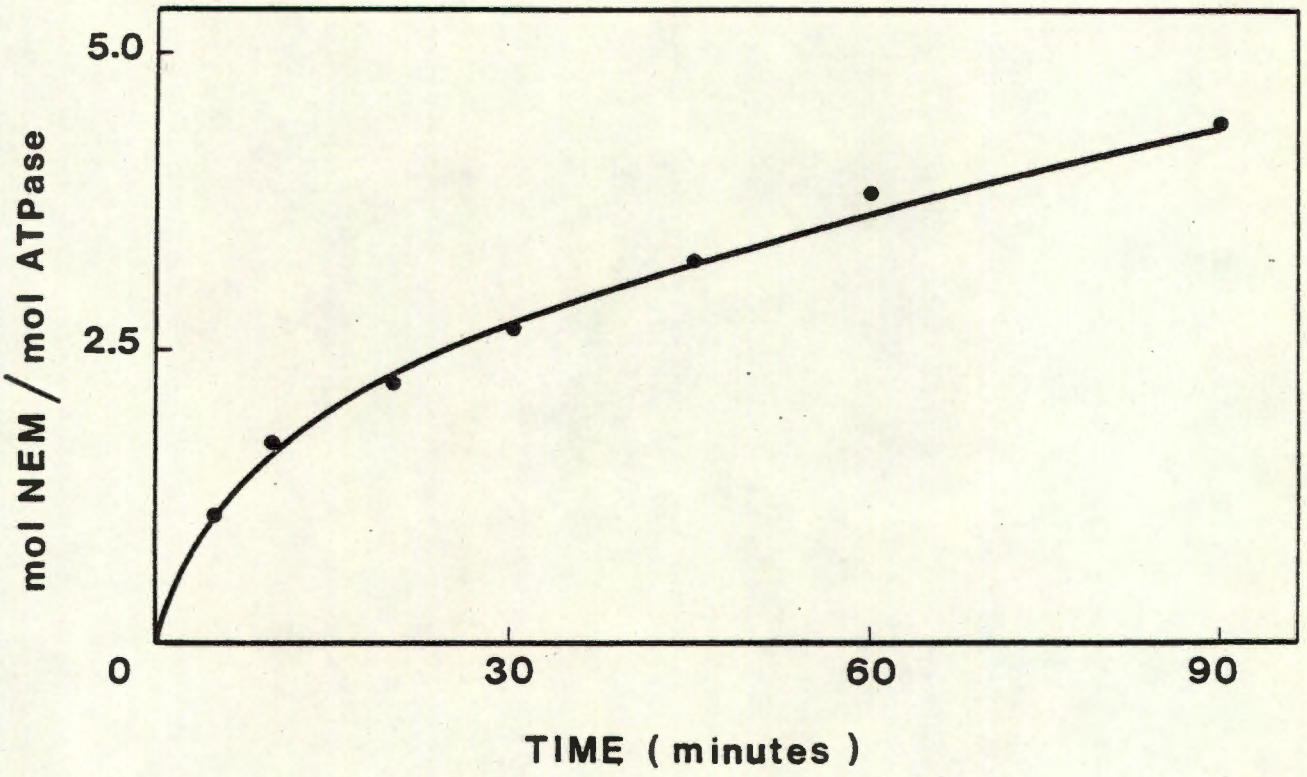


FIGURE 13 Determination of Time taken to Mask Fast Reacting -SH Groups with 5 mol NEM/mol ATPase

SR vesicles, 1.2mg/ml, were incubated in 0.1 M Tris-HCl, pH 7.4 at 25°C (Total volume = 2.0 ml). The reaction was initiated by the addition of 5 mol NEM/mol ATPase (40 µM) and 9.1 µM 1-^{14}C-NEM (800 cpm/nmol). At timed intervals aliquots were removed and the amount of protein bound label quantitated as described in "Methods".

3.4.1 Correlation of Increased -SH Group Reactivity with Calcium Transport and Ca^{2+} -ATPase Activity following EGTA-Induced Uncoupling of SR Vesicles

The effects of EGTA-uncoupling of calcium transport on the thiol group reactivity, calcium transport and Ca^{2+}-ATPase activity are shown in Figure 14. SR vesicles were preincubated with 5 mol NEM/mol ATPase and ^{14}C-NEM at 25°C for 90 min. This procedure allowed for quantitation of -SH groups blocked during this time and also provided a value for the amount of unreacted, unlabelled NEM required for determination of the specific activity of labelled NEM after addition of EGTA and a further amount of labelled NEM. EGTA and an additional amount of labelled NEM was added to the reaction mixture and the temperature was concomitantly raised to 37°C, to facilitate uncoupling. No EGTA was added to control experiments. Aliquots were removed at timed intervals to assay calcium transport (Figure 14a), Ca^{2+} -ATPase activity (Figure 14b) and -SH group reactivity (Figure 14c). The calcium transport declined rapidly in the first 10 mins. of incubation of the vesicles at 25°C. Thereafter the transport remained constant with the vesicles transporting at 60% capacity. Addition of EGTA and an increase in temperature caused a rapid decline in transport and after 5 mins the vesicles could no longer transport calcium. The ATPase activity decreased from 1.6 μ mol Pi/min/mg protein to 1.0 μ mol Pi/min/mg protein in the first 5 min. of incubation with NEM. Thereafter the

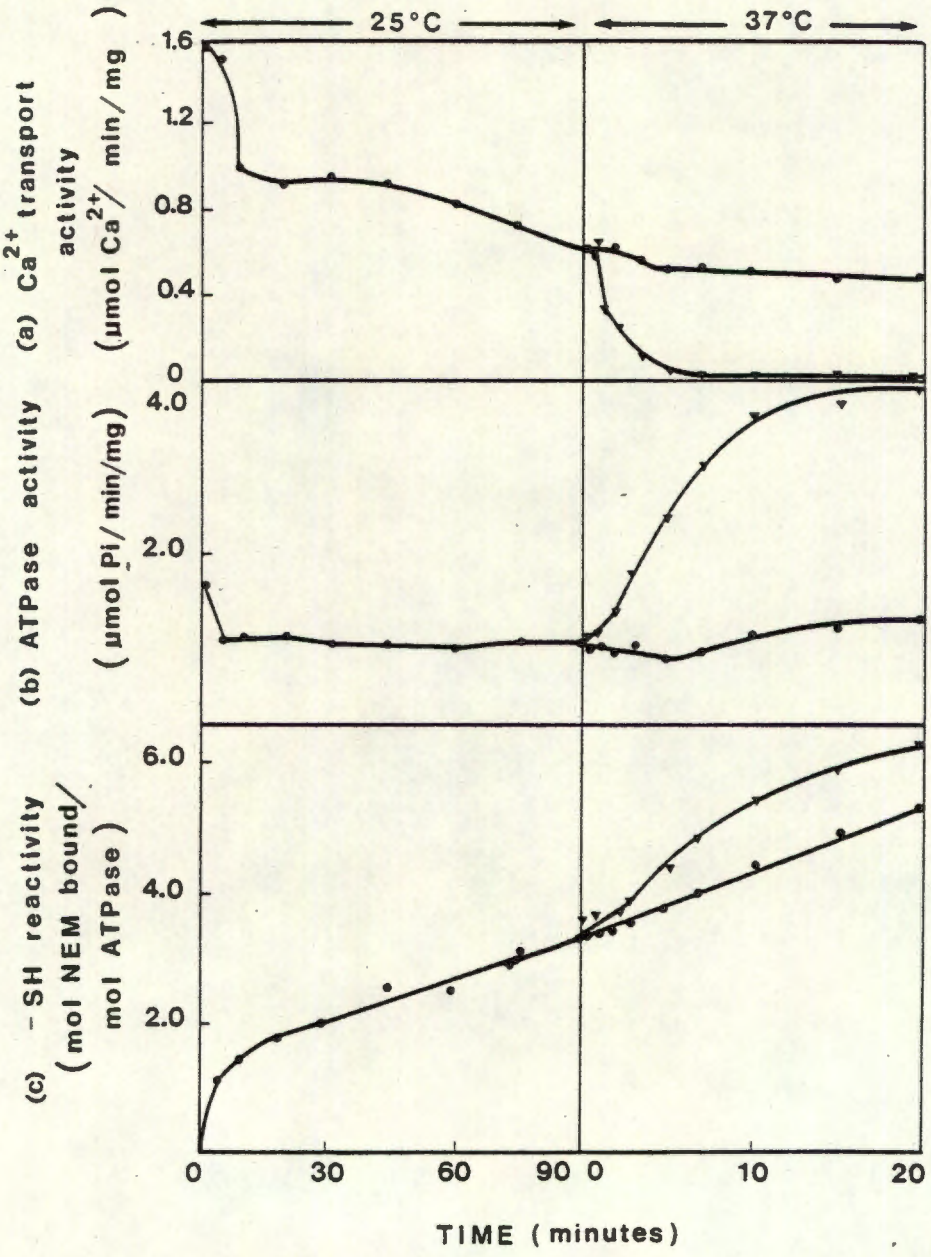


FIGURE 14 The Effect of EGTA-Treatment on the Thiol Group Reactivity, Calcium Transport Activity and Ca^{2+} -ATPase Activity of SR Vesicles

SR vesicles 2.67 mg/ml were incubated at 25°C in 11.11 mM Tris-HCl, pH 7.4, 5 mol NEM/mol ATPase (88.88 µM) and 10.125 µM 1-^{14}C-NEM (900 cpm/nmole) for 90 min. At timed intervals aliquots were removed and the bound radioactivity and ATPase

activity were calculated. The calcium transport activity was also determined using a calcium-selective electrode. All these procedures are described in "Methods". An aliquot was removed from the supernatant after TCA precipitation of the protein at 90 min to measure the amount of unlabelled, unbound NEM. This value in nmoles was required to obtain the specific activity of the labelled NEM for use in further calculations of the number of modified -SH groups following EGTA uncoupling and addition of ^{14}C-NEM.

In a separate experiment, the amount of labelled NEM incorporated into EGTA-treated and control vesicles, as well as Ca^{2+} transport activity and ATPase activity were determined by incubating SR vesicles, 2.4 mg/ml, in 10 mM Tris-HCl, pH 7.4 and 5 mol NEM/mol ATPase (80 µM) at 25°C for 90 min. After this time the reaction mixture was immediately transferred to a water bath at 37°C and EGTA (5 mM) and 1-^{14}C-NEM (7 µM; 620 cpm/nmole) were added (▼——▼). No EGTA was added to control vesicles (●——●). Aliquots were removed at timed intervals for assaying enzyme-bound radioactivity, Ca^{2+} transport activity and ATPase activity. Note that the x-axis time scales differ before and after addition of EGTA.

ATPase activity remained constant until the addition of EGTA and an increase in temperature to 37°C when ATPase activity increased markedly to approx. 175% of the original level. After 10 min. in the presence of EGTA there was no further increase in ATPase activity. The -SH group reactivity increased rapidly in the first 10 min. on incubation with NEM. Thereafter there was a gradual increase in the number of masked -SH groups until approx. 4 -SH groups/ATPase were labelled after 90 min. at 25°C. Addition of EGTA and an increase in temperature to 37°C caused a rapid increase in thiol reactivity with no further increase occurring after approx. 5 min. when approx. 1 extra -SH group/ATPase had been exposed.

There was good correlation between the decrease in Ca^{2+} transport activity and increase in reactivity of 1 -SH group/ATPase. Furthermore, the inactivation of calcium transport was accompanied by an increase in ATPase activity, which has been shown (Section 3.1) to be characteristic of the uncoupling process.

For future experiments the reaction of SR vesicles with EGTA and labelled NEM was terminated after 5 min., as further inactivation would have decreased the specificity of labelling.

3.5 Localization of the Labelled -SH Groups

The previous experiments have shown that uncoupling of calcium transport from Ca^{2+} -ATPase activity in SR vesicles resulted in approx. 1 thiol group, associated with the membrane, showing a marked increase in reactivity.

Although this may well be due to an irreversible conformational change in the (Ca^{2+}, Mg^{2+}) -ATPase, which is the main basis for this study, other possibilities need to be excluded before such a premise is acceptable.

As discussed in Section (1.2) isolated SR vesicles contain several other proteins whose -SH groups may contribute to that measured. In particular, the glycoprotein, a minor component in SR membranes, has previously been shown by Hidalgo and Thomas (1977) to possess highly reactive thiol groups, which show high reactivity towards ^{14}C-NEM. Hidalgo and co-workers have shown that the glycoprotein contains 2.5 cysteic acid residues per mol, assuming a molecular weight of 30 000. In addition to the glycoprotein there are two extrinsic proteins (Section 1.2), the high affinity calcium binding protein, (M55), molecular weight 55 000 and the calsequestrin which has a molecular weight of 44 000 and a minimum of 3 cysteic acid residues per molecule.

The following section describes a series of experiments designed to localize the -SH groups, which appear to be closely linked to energy coupling in SR membranes.

3.5.1 SDS - Polyacrylamide Disc Gel Electrophoresis of Control and EGTA-Treated SR Vesicles

In order to localize the -SH groups, radioactively-labelled during EGTA inactivation of Ca^{2+} transport, a similar procedure as previously performed was carried out. The vesicles were pre-treated with unlabelled NEM and thereafter labelled. NEM and EGTA were added and the temperature increased to 37°C to bring about inactivation of calcium transport. The labelling was terminated after 5 min by means of β-mercaptoethanol and $CaCl_2$ was added so that the free calcium concentration was approx. 100 µM. The treated and control vesicles were solubilized in SDS (1%, w/v) and the (Ca^{2+}, Mg^{2+})-ATPase identified by polyacrylamide gel electrophoresis (Figure 15). All of the protein-bound ^{14}C-label was recovered on the (Ca^{2+}, Mg^{2+})-ATPase (MW 105 000), whilst the calsequestrin, M55 and the glycoprotein were unlabelled. SR vesicles, pretreated with EGTA to inactivate calcium transport, showed approx. 2.5 fold increased labelling of the (Ca^{2+}, Mg^{2+})-ATPase, compared to control vesicles (Figure 16). EGTA did not appear to affect the mobility of the protein bands on the disc gels.

In summary, the thiol group, whose reactivity was increased as a result of uncoupling, appears to be localized on the (Ca^{2+}, Mg^{2+})-ATPase.

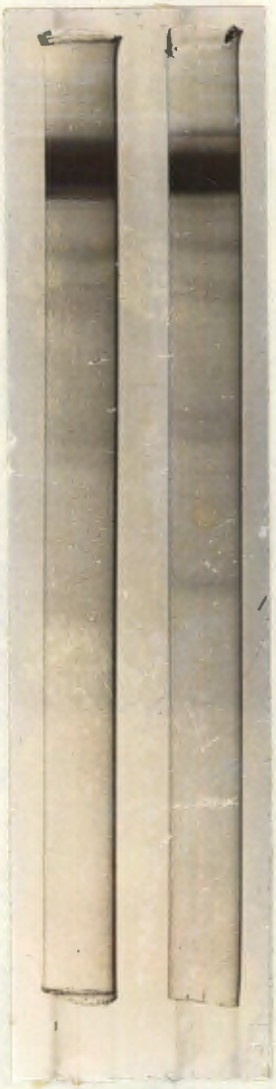


FIGURE 15 SDS-Polyacrylamide Disc Gel Electrophoresis of NEM Modified Control and EGTA-Treated SR Preparations

For details - see Figure 16.

A - control vesicles

B - EGTA-treated vesicles

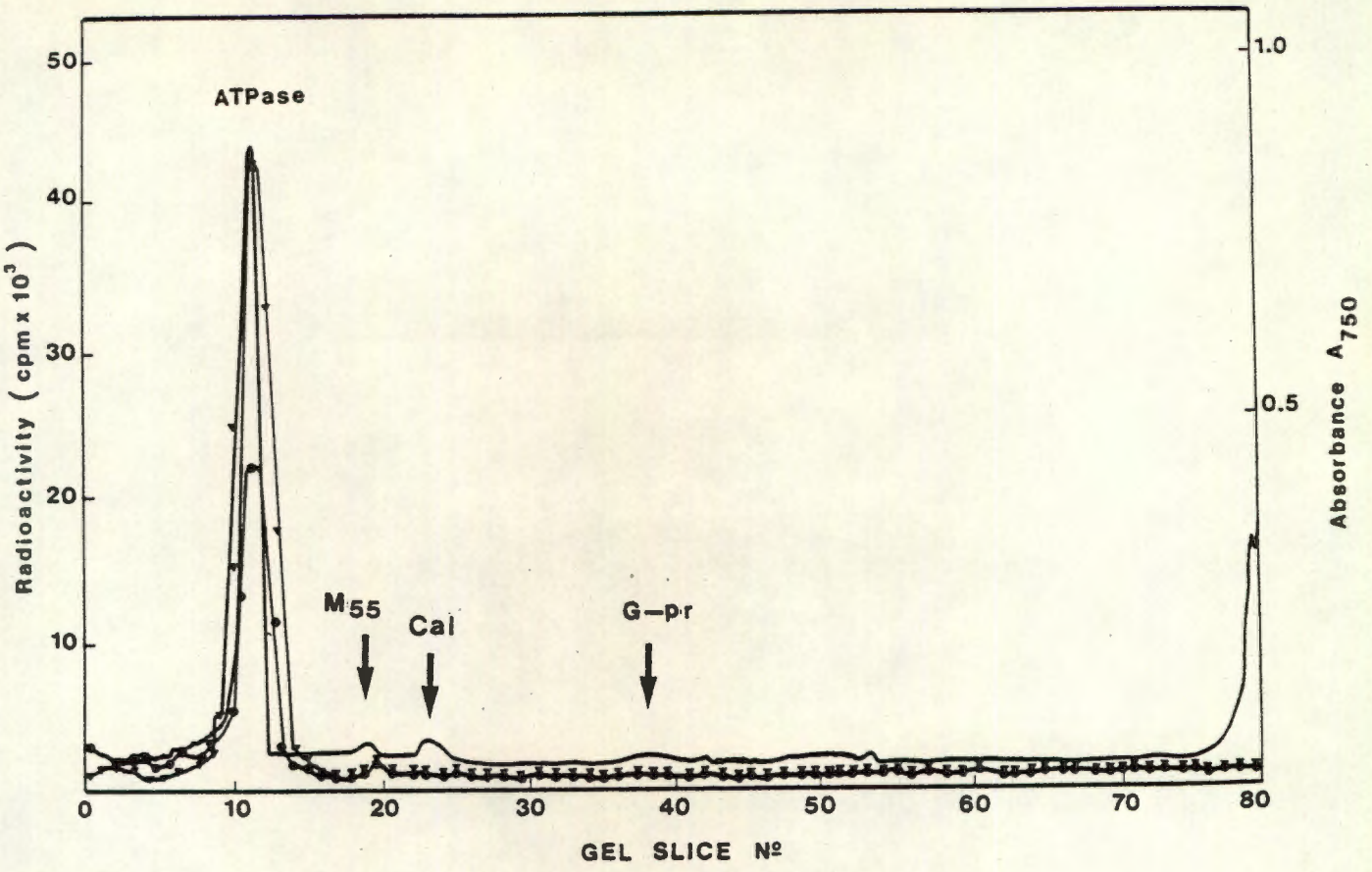


FIGURE 16 Localization of the ^{14}C-NEM Labelled -SH Group on the SR Proteins of Control and EGTA-Treated Vesicles

SR vesicles were treated in the same way as described for Figure 14. After 90 mins incubation, SR vesicles, 2.4 mg/ml, with 5 mol unlabelled NEM/mol ATPase at 25°C, were immediately placed in a water bath at 37°C and EGTA (5 mM), ^{14}C-NEM (20μM; 1750 cpm/nmol) were added (▼——▼). No EGTA was added to control vesicles (●——●). After 5 min incubation, the reaction was terminated by addition of excess β-mercaptoethanol (70 mM) and the reaction mixtures were placed on ice. EGTA, to a final concentration of 5 mM, was added to control preparations $CaCl_2$ (6.15 mM) and Tris-HCl, pH 9.8 (10 mM) were added to both preparations. This ensures that the free Ca^{2+} concentration (approx. 100 μM) remains constant in both preparations. The final pH of the reaction mixture was 7.4. The vesicles were centrifuged at 110 000 × g for 30 min in 10 mM Tris-HCl, pH 7.4 and resuspended in the same buffer. Following protein determination, SR vesicles were diluted to a concentration of 1 mg/ml with concentrated sucrose to a final concentration of sucrose of 1M. SR protein, 70 μg, was then solubilized in 1% (w/v) SDS and applied to SDS-polyacrylamide disc gels according to the method of Weber and Osborne (1969) (described in "Methods"). Following staining in Coomassie blue, and destaining, gels were scanned in a densitometer (Vitatron), sliced and the radioactivity in each gel slice was measured as described in "Methods".

Absorbance at 750 nm (——).

3.5.2 Distribution of Radioactivity in Tryptic Peptides from Control and EGTA-Treated SR Vesicles following Labelling with ^{14}C-NEM

Limited proteolysis of the (Ca^{2+}, Mg^{2+})-ATPase with trypsin produces smaller subfragments (Section 1.2) which have been assigned specific functions (Inesi and Scales, 1974; Thorley-Lawson and Green, 1973 and 1975; and Stewart and MacLennan, 1974). In order to endeavour to locate the specific domain of the ATPase, where the possible change in conformation is occurring during the process of EGTA-uncoupling, the reactive thiol group was labelled as previously described. The SR vesicles were then treated with trypsin, solubilized and electrophoresed on SDS-disc gels (Figure 17). Limited trypsinization of the (Ca^{2+}, Mg^{2+})-ATPase (referred to as *single cleavage*) produced four subfragments of molecular weights 55 000, 45 000, 30 000 and 20 000 respectively. More extensive trypsinization, (referred to as *double cleavage*) resulted in a significant decrease in the amount of 55 000 dalton subfragment and an increase in the 30 000 dalton and 20 000 dalton subfragments (Figures 17, 18 and 19). These findings are similar to those previously described by Inesi and Scales (1974), Thorley-Lawson and Green (1973 and 1975) and Stewart and MacLennan (1974), however, the single and double cleavages appeared to be more extensive in our preparations. The previous studies have shown that single cleavage results only in the formation of the 55 000 dalton and 45 000 dalton subfragments whilst double cleavage produces well

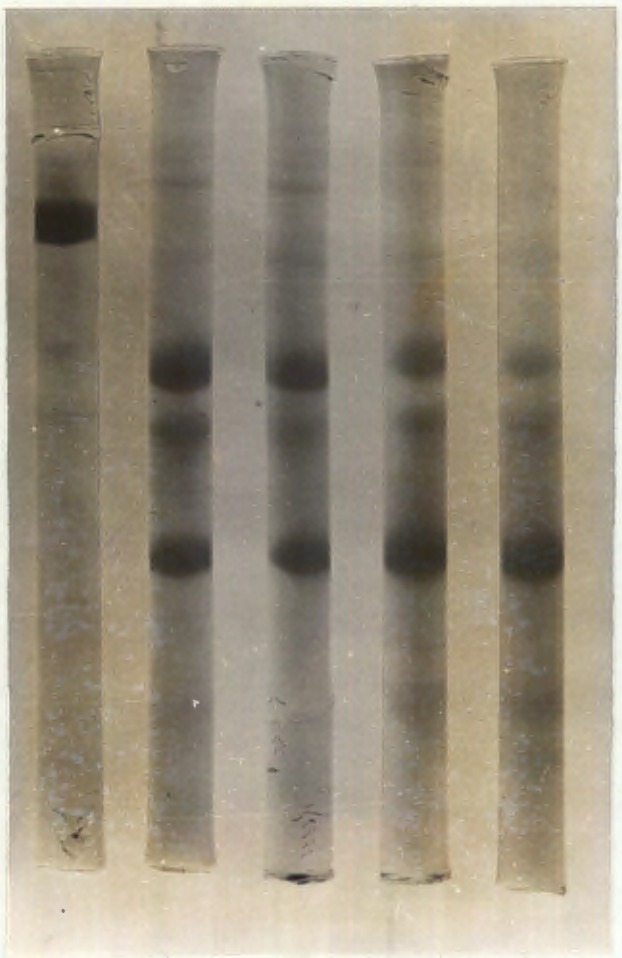


FIGURE 17 SDS-Polyacrylamide Disc Gel Electrophoresis of ^{14}C-NEM Modified Control and EGTA-Treated SR Vesicles Digested with Trypsin

For details - see Figures 18 and 19

A - SR proteins
B - single tryptic cleavage of control vesicles
C - single tryptic cleavage of EGTA-treated vesicles
D - double tryptic cleavage of control vesicles
E - double tryptic cleavage of EGTA-treated SR vesicles

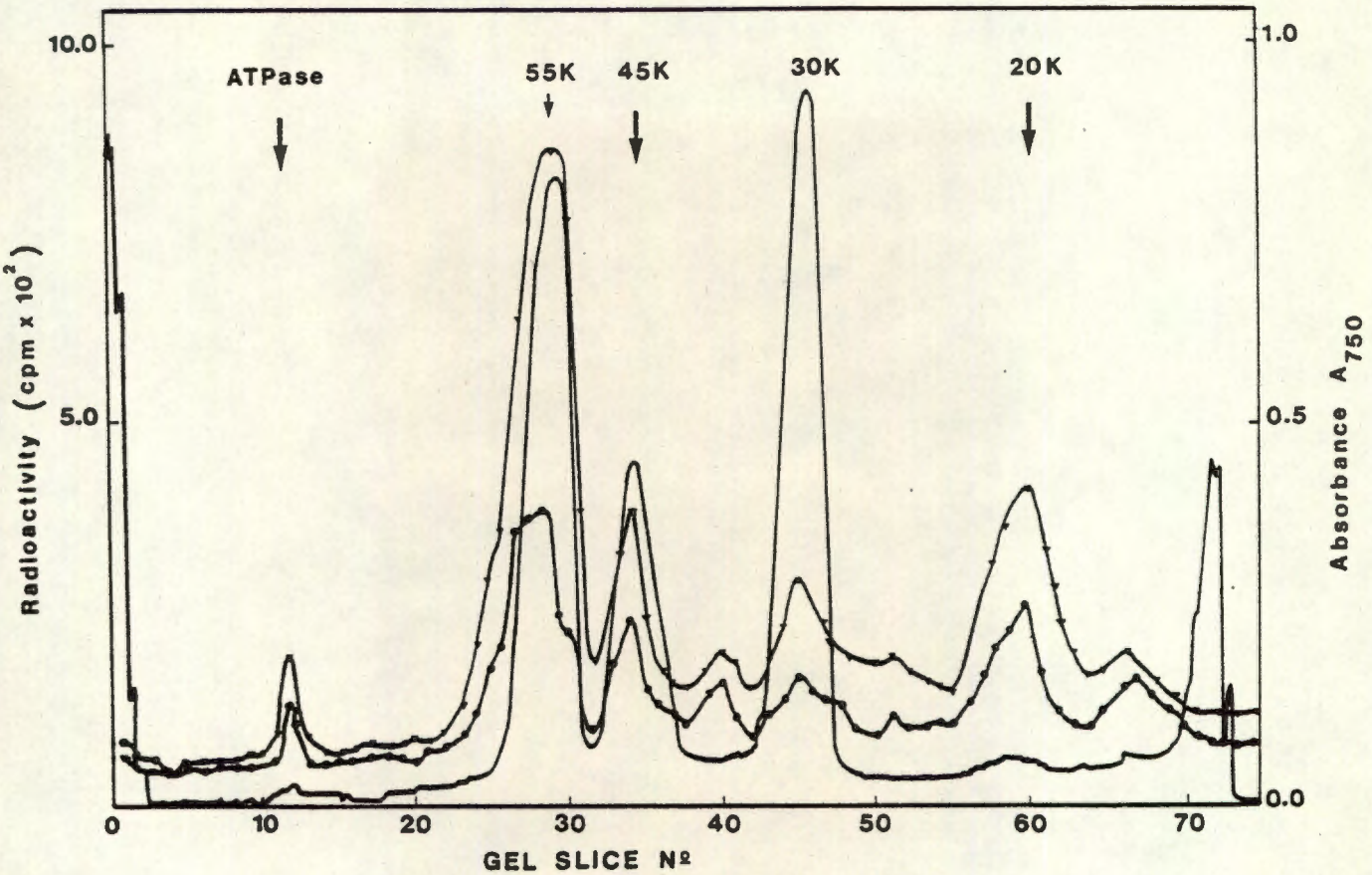


FIGURE 18 Distribution of the ^{14}C-NEM Labelled-SH Groups on the Tryptic Subfragments of the (Ca^{2+}, Mg^{2+}) -ATPase, Following Single Tryptic Cleavage of Control and EGTA-Treated SR Vesicles

SR vesicles were treated in exactly the same manner as for Figure 16. After centrifugation, the vesicles were suspended to a concentration of 1 mg/ml in 1 M sucrose and trypsin was added to a final concentration of 1000:1 protein to trypsin (single cleavage). The reaction mixture was incubated at 25°C for 5 min and digestion was terminated by addition of soyabean trypsin inhibitor at twice the concentration of trypsin. SR vesicles were then centrifuged at 110 000 × g for 30 min in 10 mM Tris-HCl, pH 7.4. After resuspending the vesicles in the same buffer, 140 µg of protein was solubilized in 1% (w/v) SDS and layered onto SDS polyacrylamide disc gels. The remaining procedures of densitometric scanning, slicing and assaying of radioactivity were carried out as in (Figure 13) and as described in "Methods".

Absorbance at 750 nm (——).

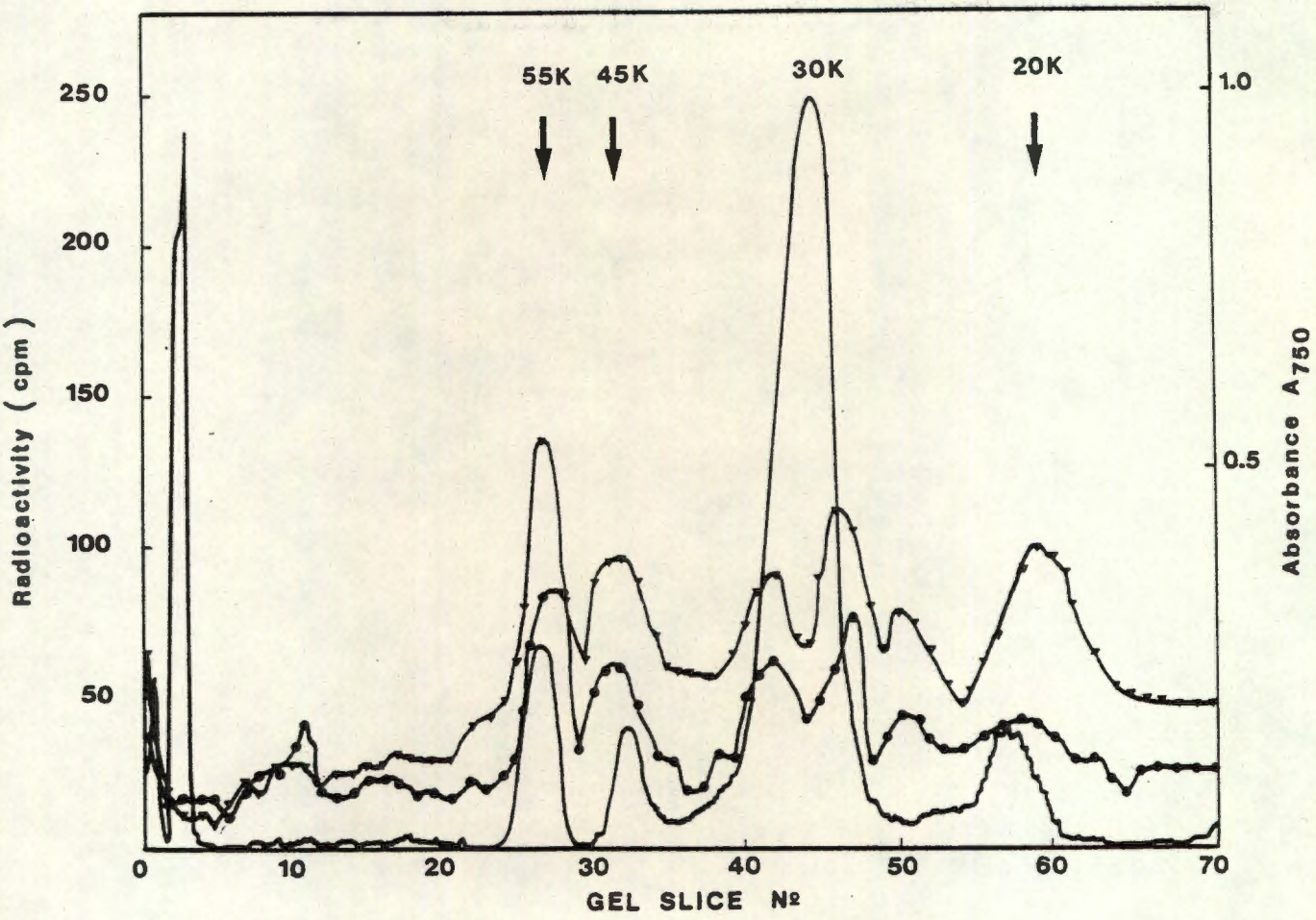


FIGURE 19 Distribution of ^{14}C-NEM Labelled -SH Groups on Tryptic Subfragments of the (Ca^{2+}, Mg^{2+}) -ATPase Following Double Tryptic Cleavage of Control and EGTA-Treated SR Vesicles

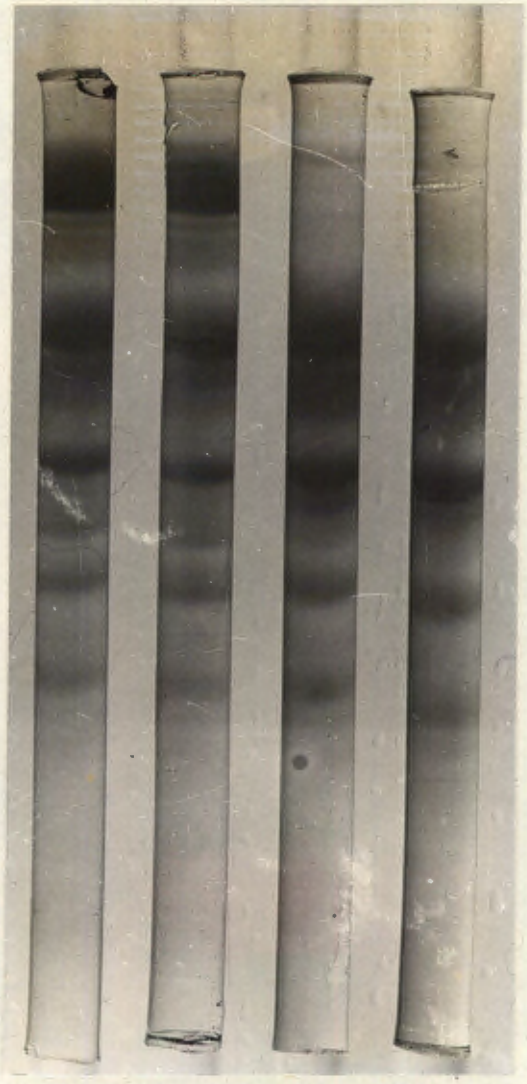
This experimental procedure is essentially similar to that described for Figure 18, except that trypsin was added to a final concentration of 1:200 trypsin to protein (double cleavage). The reaction mixture was incubated at 25°C for 15 min, thereafter soyabean trypsin inhibitor was added to a final concentration of 1:2 trypsin to inhibitor. The remainder of the experiment was identical to that described for Figure 18.

relative increase in label appeared to be localized on the 55 000 dalton and the 20 000 dalton subfragments. The latter is a product of the cleavage of the 55 000 dalton subfragment.

In this procedure, designed to localize the NEM modified -SH groups in SR membranes, the material analysed is that which sedimented at 110 000 x g for 30 mins. This material was well washed prior to isolation of the tryptic fragments on the disc gels. These fractions thus represent peptides which remain bound to the SR membranes.

The previous experiments were repeated several times and although there were general similarities in the labelling of the tryptic subfragments, the digestion patterns were somewhat variable. For example, single and double tryptic digestion were often extensive and subfragments of molecular weight less than 20 000 were present (Figures 20 and 21). The soyabean trypsin inhibitor did not appear to completely inhibit the tryptic digestion and for this reason the trypsin and trypsin inhibitor were removed immediately by centrifugation following trypsin treatment. This procedure prevented extensive trypsinization occurring.

A further significant observation was that following digestion, and particularly double tryptic cleavage, there was loss of protein-bound label. One of the possibilities considered was that significant quantities of peptide were released into the medium as soluble material following the high speed centrifugation step. This material could have been either free peptides or peptides associated with lipid. Selective solubilization of tryptic fragments would obviously have

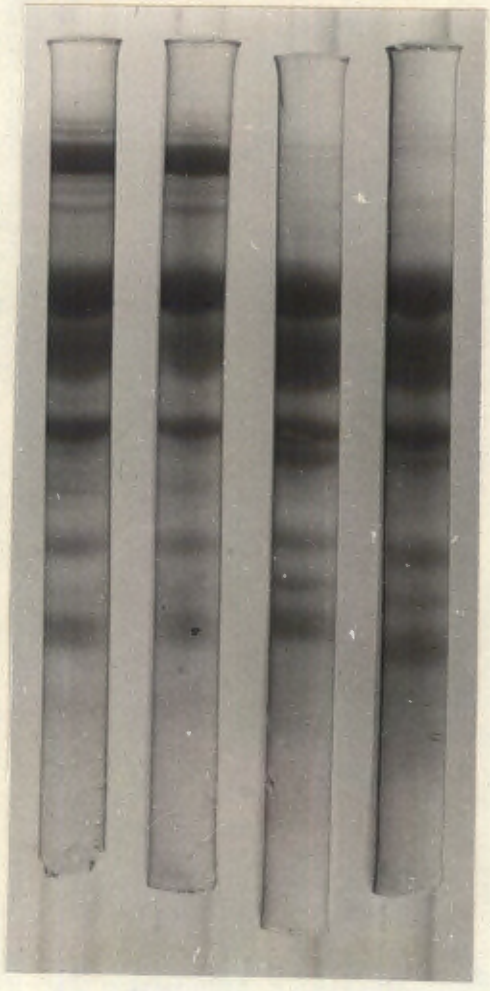


A B C D

FIGURE 20 SDS-Polyacrylamide Disc Gel Electrophoresis of ^{14}C-NEM Modified Control and EGTA-Treated SR Vesicles, Digested with Trypsin

For details - see Figures 18 and 19.

B - single tryptic cleavage of control vesicles
C - single tryptic cleavage of EGTA-treated vesicles
D - double tryptic cleavage of control vesicles
E - double tryptic cleavage of EGTA-treated vesicles



A B C D

FIGURE 21 SDS-Polyacrylamide Disc Gel Electrophoresis of ^{14}C-NEM Modified Control and EGTA-Treated SR Vesicles, Digested with Trypsin

For details - see Figures 18 and 19.

B - single tryptic cleavage of control vesicles
C - single tryptic cleavage of EGTA-treated vesicles
D - double tryptic cleavage of control vesicles
E - double tryptic cleavage of EGTA-treated vesicles

defined 30 000 dalton and 20 000 dalton subfragments.

In the present experiments single tryptic cleavage resulted in all of the fragments being labelled in control SR vesicles. EGTA-treated vesicles showed an increase in protein-bound label on all the fragments (Figure 18). The ratios of labelling of the fragments in control and EGTA-treated vesicles are shown in Table 5. The species which showed the greatest increase in radioactive label were the ATPase, the 55 000 dalton and the 20 000 dalton fragments.

TABLE 5 Ratios of Labelling of Polypeptide Species in Control and EGTA-Treated Vesicles

Polypeptide Species	Ratio i.e. ^{14}C(EGTA)/^{14}C (Control)
ATPase (MW 105 000)	2.20
Tryptic Fragments	
55 000 daltons	2.35
45 000 daltons	1.15
30 000 daltons	1.32
20 000 daltons	2.13

Similar results were obtained with the double tryptic cleavage (Figure 19). Although there was increased labelling in all the fragments, the 20 000 dalton fragment showed the greatest increase in labelling.

Thus, there appeared, in general, to be a variable pattern of labelling with the EGTA-treated vesicles, with increased labelling on all the fragments. The greatest

altered the ratio of remaining membrane-bound material.

Inesi and Asai (1968) have shown that under conditions similar to those employed here to produce the double tryptic cleavage products, approx. 80% of the protein remains associated with the SR membrane following trypsinization. Our studies, reported in a forthcoming section (3.5.5) did, however, show that, following NEM modification and tryptic cleavage, a considerable fraction of membrane protein is rendered soluble and there is significant loss of protein-bound label into the medium.

The possibility was also considered that some of the labelled peptides noted during polyacrylamide disc gel electrophoresis were autodigestion products of trypsin or trypsin inhibitor. This was thought to be unlikely in view of the low concentrations of trypsin and trypsin inhibitor employed (maximum 1% of SR protein) and also the fact that autodigestion products of trypsin are soluble and would not be recovered in the 110 000 × g sediment which was analysed. A further factor was that β-mercaptoethanol was added prior to trypsinization and this would react with all the ^{14}C-labelled NEM.

A similar experiment was therefore performed as previously described for Figure 19, however, no SR vesicles were included in the procedure and an excess of the reaction mixture was layered onto disc gels, in order to visualize trypsin (molecular weight 23 300) and trypsin inhibitor bands. This control experiment (Figure 22) confirmed that there was no detectable radioactivity in the EGTA-treated and control sedimentable preparations.

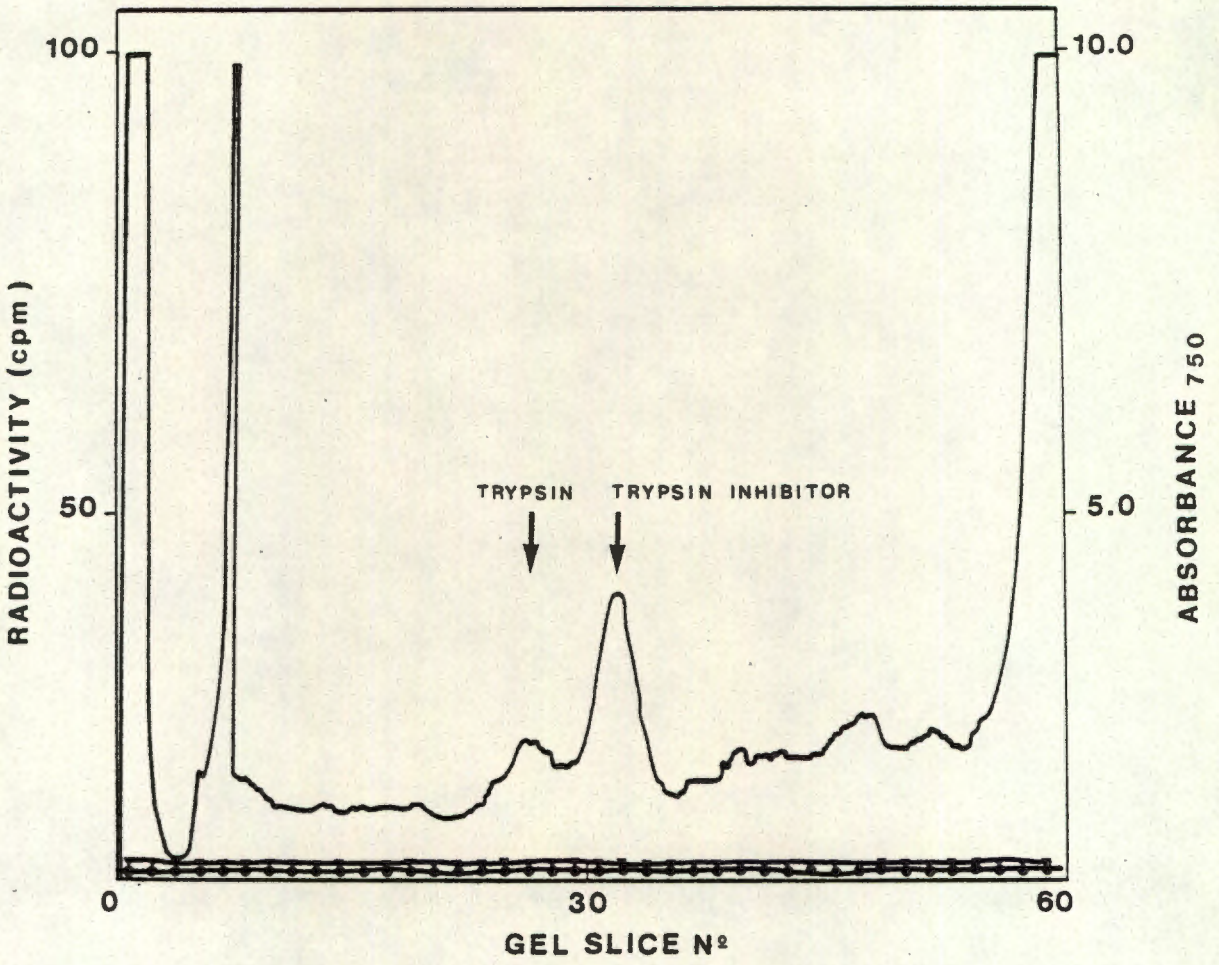


FIGURE 22 Autodigestion of Trypsin

This method is similar to that described for Figure 19, except that no SR vesicles were included in both EGTA-treated (▼——▼) and control (●——●) experiments. The centrifugation step following addition of trypsin and inhibitor was not required and 250 µl of reaction mixture was layered onto each gel. Densitometric scanning, slicing, and counting of radioactivity are described in "Methods".

The validity of kinetic data using NEM as a -SH reagent is based upon the assumption that excess β-mercaptoethanol used to stop the reaction, is completely effective. This was confirmed in an experiment in which excess β-mercaptoethanol was added to SR vesicles prior to the addition of ^{14}C-NEM (Figure 23). Under these conditions β-mercaptoethanol completely inhibited binding of ^{14}C-label to SR membrane proteins.

3.5.3 The Possible Effects of NEM Modification of Tryptic Fragments on their Electrophoretic Mobility, as Determined by SDS-Polyacrylamide Disc Gel Electrophoresis

Identification of tryptic subfragments of the (Ca^{2+},Mg^{2+})-ATPase was based on their electrophoretic mobility (Stewart and MacLennan, 1974). An experiment was designed to determine the effect of NEM-modification on the electrophoretic mobility of the tryptic subfragments. SR vesicles were trypsinized to obtain single and double tryptic cleavage products and then treated with 5 mol and 20 mol NEM/mol ATPase, respectively. The tryptic subfragments and their NEM treated derivatives showed identical mobilities (Figure 24). In addition staining of tryptic fragments with Coomassie blue was not affected by NEM treatment. Thus it was assumed that NEM modification did not alter the electrophoretic mobility of the subfragments.

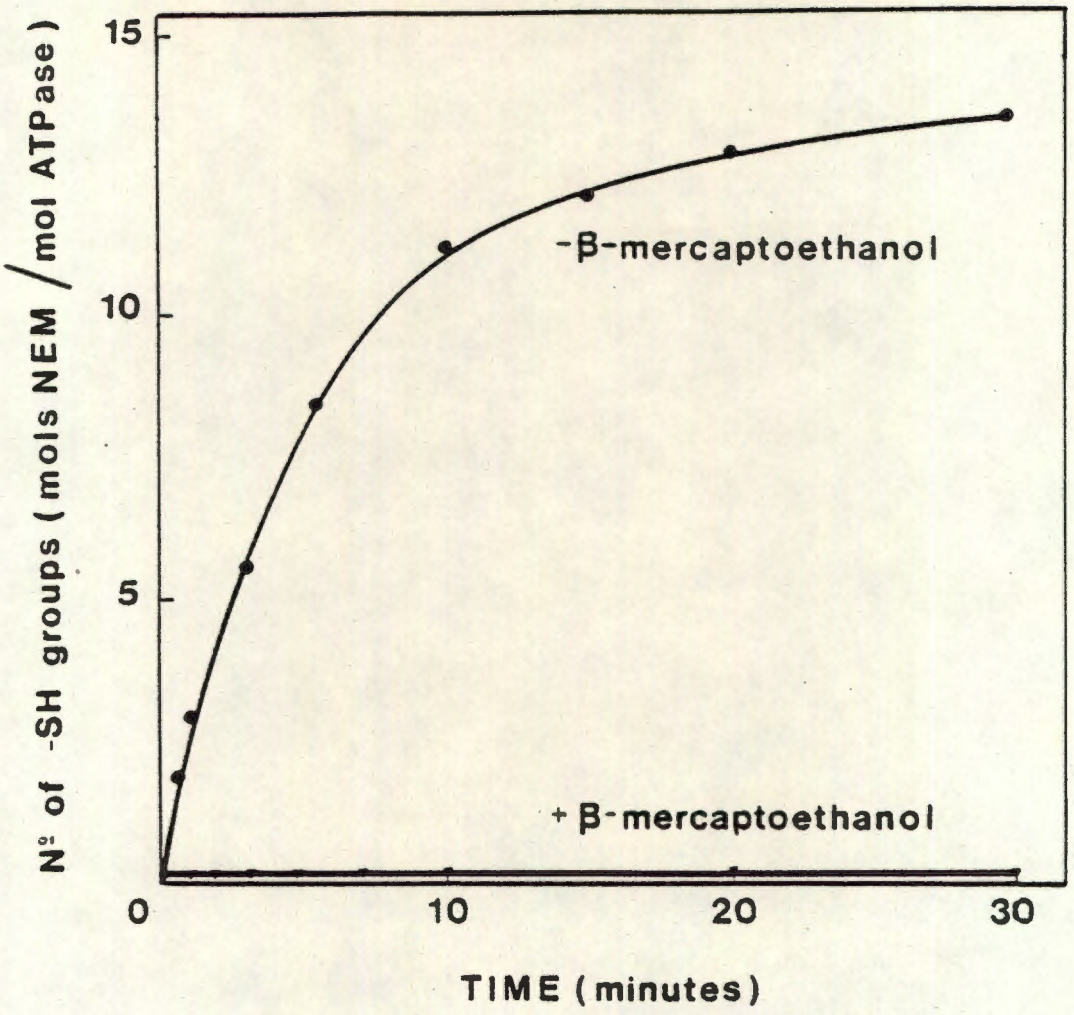


FIGURE 23 Effect of β-Mercaptoethanol on NEM-Modification of SR Vesicles

SR vesicles, 2.4 mg/ml, were incubated in 10 mM Tris-HCl, pH 8.0 and excess β-mercaptoethanol (120 mM) for 1 min. at 25°C. Thereafter 2 mM NEM and 20 μM ^{14}C-NEM (1750 cpm/nmol) were added. At timed intervals 125 μl aliquots were removed and the amount of radioactive label bound to the protein quantitated (▼——▼) as described in "Methods". Control experiment was performed as above in the absence of β-mercaptoethanol (●——●).

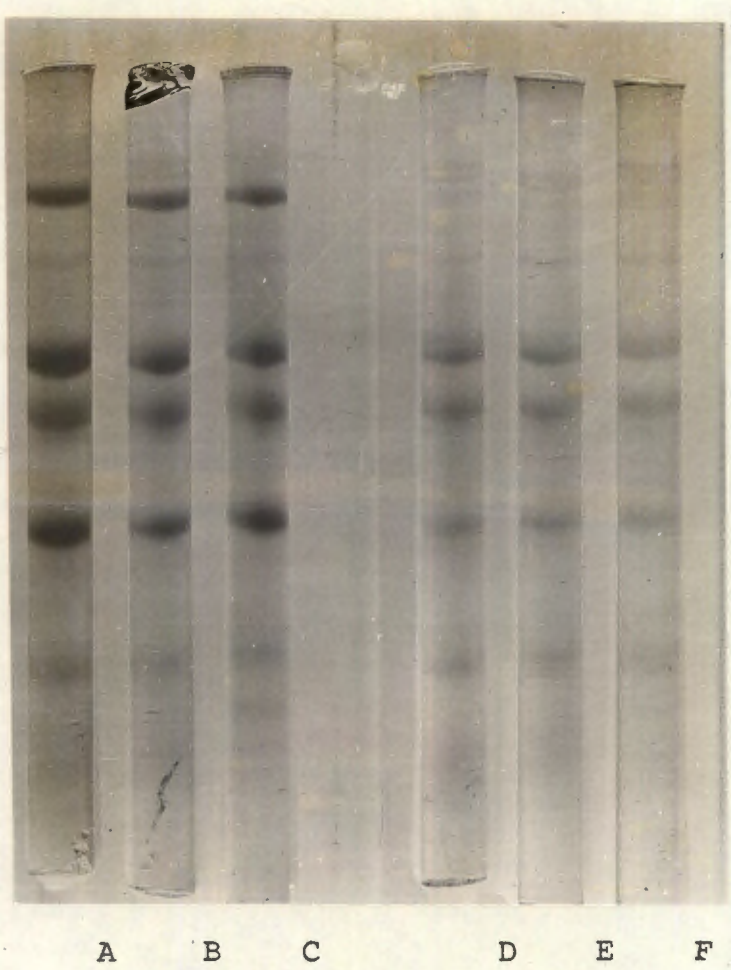


FIGURE 24 SDS-Polyacrylamide Disc Gel Electrophoresis of the Tryptic Subfragments of the (Ca^{2+}, Mg^{2+}) -ATPase Modified with NEM

SR vesicles, 1 mg/ml, were incubated in 10 mM Tris-HCl, pH 7.4 and 1 M sucrose at 25°C. Trypsin was added to a final concentration of 1000:1 (single cleavage) and 200:1 (double cleavage) protein to trypsin and the reaction allowed to proceed for 5 and 15 mins respectively. Thereafter soyabean trypsin inhibitor was added at twice the concentration of trypsin. The vesicles were centrifuged at 110 000 × g for 30 mins in 10 mM Tris-HCl, pH 7.4. Tryptic cleaved SR vesicles, at a final concentration of 1 mg protein/ml, were modified with 5 mol NEM/mol ATPase and 20 mol NEM/mol ATPase respectively at 25°C for 90 min, after which time excess β-mercaptoethanol was added (140 mM). 100 µg of SDS-solubilized protein was applied to SDS-polyacrylamide gels as described in "Methods".

A - single tryptic cleavage of SR

B - single tryptic cleavage of SR vesicles and subsequent modification with 5 mol NEM/mol ATPase.

C - single tryptic cleavage of SR vesicles and subsequent modification with 20 mol NEM/mol ATPase.

D - double tryptic cleavage of SR vesicles

E - double tryptic cleavage of SR vesicles and subsequent modification with 5 mol NEM/mol ATPase

F - double tryptic cleavage of SR vesicles and subsequent modification with 20 mol NEM/mol ATPase

3.5.4 The Possible Effects of NEM Modification of SR Vesicles on Tryptic Cleavage Patterns

The possible effects of NEM modification of SR vesicles on subsequent tryptic digestion was also investigated. SR vesicles, incubated with 5 mol and 20 mol NEM/mol ATPase, respectively, were digested with trypsin (Figure 25). The cleavage products remained the same as previously described and the mobility of the subfragments were unchanged. However, NEM modification does appear to increase the susceptibility of the (Ca^{2+}, Mg^{2+}) -ATPase to tryptic digestion. In the single tryptic cleavage, for example, increasing the concentration of NEM resulted in an increase of the subfragment of MW 30 000. The double tryptic cleavage pattern showed decreased amounts of 55 000, 45 000 and 20 000 dalton fragments with increasing concentration of NEM. The 30 000 dalton subfragment appeared as a doublet and in the presence of 5 mol NEM/mol ATPase. In general the 45 000 dalton fragment appeared to be more diffuse when trypsinization was carried out following NEM modification and the double tryptic cleavage was more extensive with increasing concentrations of NEM.

In summary, NEM modification does not appear to affect the tryptic cleavage patterns or the mobility of the tryptic subfragments, however, it does appear to affect the extent of trypsinization.

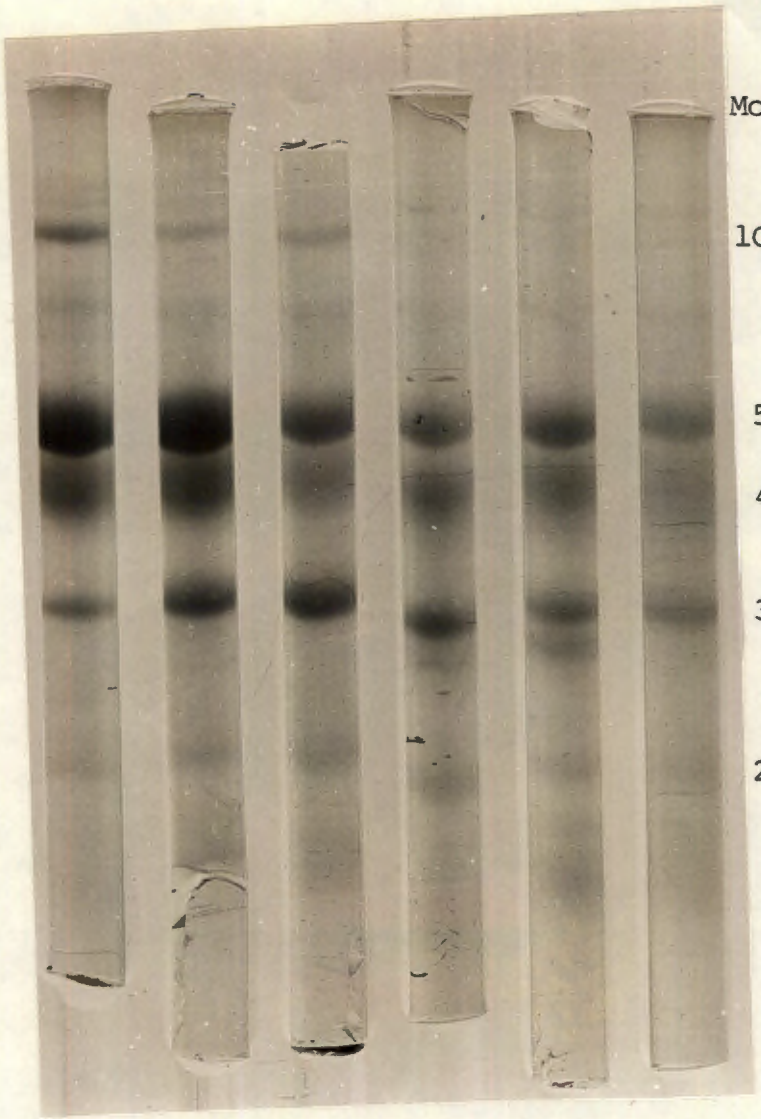


FIGURE 25 SDS-Polyacrylamide Disc Gel Electrophoresis of NEM Modified SR Vesicles Digested with Trypsin

SR vesicles, 2.4 mg/ml, were incubated in 10 mM Tris-HCl, pH 7.4 with 5 mol and 20 mol NEM/mol ATPase, respectively at 25°C for 90 min. Thereafter the SR vesicles were diluted to a concentration of 1 mg/ml with concentrated sucrose (final concentration 1 M), 10 mM Tris-HCl, pH 7.4. Trypsin was added to a final concentration of 1000:1 and 200:1 protein to trypsin for the single and double tryptic cleavages, respectively. The vesicles were incubated for 5 and 15 mins, respectively and then soyabean trypsin inhibitor was added at twice the concentration of trypsin. The vesicles were centrifuged at 110 000 × g for 30 min. in 10 mM Tris-HCl, pH 7.4 and resuspended in the same buffer. 100 µg of SDS-solubilized protein was added to each gel as described in "Methods".

A - SR vesicles digested with 1000:1 protein to trypsin

B - SR vesicles modified with 5 mol NEM/ mol ATPase and digested with 1000:1 protein to trypsin

C - SR vesicles modified with 20 mol NEM/mol ATPase and digested with 1000:1 protein to trypsin

D - SR vesicles digested with 200:1 protein to trypsin

E - SR vesicles modified with 5 mol NEM/mol ATPase and digested with 200:1 protein to trypsin

F - SR vesicles modified with 20 mol NEM/mol ATPase and digested with 200:1 protein to trypsin

3.5.5 Solubilization of NEM Modified Membrane-Bound Protein during Tryptic Digestion of Control and EGTA-Treated SR Vesicles

These and the following experiments were carried out to determine the extent of release of peptide material and ^{14}C-label following tryptic digestion of ^{14}C-NEM modified SR membranes. Partial characterization of solubilized tryptic fragments was also attempted.

Results are shown in Table 6. Mild tryptic digestion (single cleavage) of control labelled SR vesicles resulted in loss of approx. 25% of protein and 35% of ^{14}C-label from the membrane bound fraction. More extensive trypsinization (double cleavage) resulted in loss of approx. 40% of protein and 75% of ^{14}C-label. In unmodified SR vesicles subjected to both trypsin concentrations (Table 7) 20% of protein was lost in the single cleavage and 25% of protein in the double cleavage, respectively. These results agree with the findings of Inesi and Asai (1968) who found a decrease in turbidity of SR vesicles undergoing trypsin digestion. After prolonged centrifugation of these vesicles, they found that the amount of insoluble protein had decreased. At protein to trypsin concentrations of 100:1 they found 20% loss of protein after incubation at 25°C for 15 mins. Yoshida and Tonomura (1976) found that when SR vesicles, modified with 2 mol of bound NEM per 10^5 g of SR protein, was subjected to similar trypsin concentrations as stated above for 1 hour, 95% of the protein remained insoluble. However, 50% of the bound NEM was

TABLE 6 Recoveries of Protein and ^{14}C - Label from NEM Modified Protein Following Trypsinization

FRACTION	CONTROL VESICLES				EGTA-TREATED VESICLES			
	pr (mg)	% pr (±n)	cpm×10^6	% cpm (±n)	pr (mg)	%pr (±n)	cpm×10^6	% cpm (±n)
SR + ^{14}C-NEM	6.00	100.0	3.50	100.0	6.00	100.0	3.60	100.0
Pellet + ^{14}C-NEM + 2 washes	4.86	81.0	1.07	30.7	4.98	83.0	1.76	49.0
Supernatant	-	-	0.033	0.93	-	-	0.03	0.81
SR + ^{14}C-NEM	2.00	100.0	cpm×10^5 4.40	100.0	2.00	100.0	cpm×10^5 7.07	100.0
Pellet + single cleavage + spin	1.46	73.0 ± 1.06	2.73	62.0 ± 3.54	1.39	69.5 ± 1.27	4.31	61.0 ± 3.18
Supernatant	0.42	14.0 ± 3.28	1.49	33.6 ± 3.81	0.45	20.5 ± 3.63	2.50	35.4 ± 4.02
Pellet + double cleavage + spin	1.06	53.0 ± 1.55	0.52	11.8 ± 4.13	1.02	51.0 ± 1.51	0.72	10.2 ± 2.83
Supernatant	0.88	44.0 ± 7.2	3.30	75.0 ± 4.42	0.89	44.5 ± 7.56	5.40	76.4 ± 4.81

n = SD of 5 experiments pr = protein For details see following page.

TABLE 6 Recoveries of Protein and ^{14}C-Label from NEM Modified Protein Following Trypsinization

SR vesicles, 2.4 mg/ml, were incubated in 10 mM Tris-HCl, pH 7.4 and 5 mol NEM/mol ATPase for 90 min at 25°C. The temperature was immediately raised to 37°C and EGTA (5 mM) and ^{14}C-NEM (33 μM, 3000 cpm/nmol) were added to the EGTA-treated vesicles. No EGTA was added to control vesicles. After 5 min excess β-mercaptoethanol was added (100 mM) and the reaction mixtures were placed on ice. EGTA (5 mM) was added to control preparations. $CaCl_2$ (6.15 mM) and Tris-HCl, pH 9.8 (9.45 mM) were added to both preparations. The vesicles were washed twice at 110 000 × g for 30 min in 10 mM Tris-HCl, pH 7.4 and resuspended in the same buffer. Following each centrifugation step the amount of protein was determined. The supernatants were retained and aliquots were removed, in duplicate, from these and the resuspended protein to determine the total amount of radioactivity present. The modified SR vesicles at a final concentration of 1 mg/ml in 1M sucrose were treated with 1000:1 (single cleavage) and 200:1 (double cleavage) protein to trypsin for 15 min and 5 min respectively. The vesicles were centrifuged at 110 000 × g for 30 min in 10 mM Tris-HCl, pH 7.4. The amount of protein in the pellets and supernatants was determined as well as the total radioactivity in both fractions. The experiment was repeated five times and standard deviations (SD) of the recovery of protein and radioactivity following trypsinization were calculated.

TABLE 7

Recovery of Insoluble Protein from Unmodified SR Vesicles Following Trypsin Treatment

FRACTION	UNMODIFIED PROTEIN		^{14}C-NEM MODIFIED PROTEIN	
	protein (mg)	% protein (±n)	protein (mg)	% protein (±n)
SR Vesicles	2.00	100.0	2.00	100.0
SR incubated at 25°C for 15 min (Control + spin)	1.75	87.5	-	-
Single tryptic cleavage + spin	1.55	77.5 ± 1.23	1.46	73.0 ± 1.06
Double tryptic cleavage + spin	1.50	75.0 ± 1.31	1.06	53.0 ± 1.55

SR vesicles, 1 mg/ml were incubated in 10 mM Tris-HCl, pH 7.4 and 1M sucrose at 25°C. Trypsin was added to a final concentration of 1000:1 (single cleavage) and 200:1 (double cleavage) protein to trypsin for 5 & 15 min respectively. The digestion was terminated by the addition of soyabean trypsin inhibitor at twice the concentration of trypsin. No trypsin or inhibitor was added to control vesicles which were incubated at 25°C for 15 mins. The vesicles were centrifuged at 110 000 xg for 30 min. in 10 mM Tris-HCl, pH 7.4 and the amount of protein in the pellet determined.

(n = SD of 5 experiments)

solubilized by this treatment with trypsin. EGTA-treated vesicles showed similar percentage losses of protein and ^{14}C-label following trypsinization compared to control vesicles. However, there was approximately twice the amount of bound ^{14}C-label in EGTA-treated vesicles due to the exposure of an extra -SH group during uncoupling.

The nature of released peptide material and radioactive label was further examined using larger quantities of SR vesicles. Bulk quantities of SR vesicles were modified with ^{14}C-NEM, EGTA-treated and then trypsinized to obtain the double cleavage products. The amount of protein and the radioactivity in the supernatant (Ts), and pellet, (Tp), following high speed centrifugation was assayed (Table 8). The nature of this protein was also investigated by SDS-polyacrylamide slab gel electrophoresis. The supernatant, which contained the bulk of the label, was then concentrated by ultrafiltration. This procedure resulted in an increase in turbidity of the solution, presumably due to precipitation of soluble material being rendered insoluble. This insoluble material was removed by centrifugation and the resulting supernatant was reconcentrated by ultrafiltration. This concentrate and the original precipitate were investigated by means of SDS-polyacrylamide slab gel electrophoresis and the radioactivity was detected by scanning in a gas flow proportional counter.

SDS-polyacrylamide slab gels of the pellet, (Tp), and supernatant, (Ts), (Figure 26), showed that the peptide patterns and their mobilities were identical in control and EGTA-treated

TABLE 8

RECOVERY OF PROTEIN AND ^{14}C-LABEL IN THE SUPERNATANT AND ITS CONCENTRATED FRACTIONS FOLLOWING TRYPSINIZATION

	FRACTION	CONTROL VESICLES				EGTA-TREATED VESICLES			
		pr(mg)	% pr	cpm	% cpm	pr(mg)	% pr	cpm	% cpm
1.	NEM modified SR + trypsin (double cleavage)	20.00	100.0	9.18×10^5	100.00	20.00	100.0	1.81×10^6	100.0
2.	Pellet + spin T_p (i.e. TpC and TpE)	6.10	30.5	4.71×10^4	5.1	5.36	26.8	9.20×10^4	5.1
3.	Supernatant + spin Ts (i.e. TsC and TsE)	13.60	68.0	7.17×10^5	78.1	13.26	66.3	1.42×10^6	78.4
4.	Supernatant concentrated using PM10 filters Filtrate(Ts Fil)	-	-	1.79×10^5	25.0	-	-	3.83×10^5	27.1
5.	Concentrate, TsConc	-	-	3.24×10^5	45.2	-	-	6.58×10^5	46.4
6.	Concentrate, TsConc-spin at 230 000 xg for 30 min. Pellet, TsConcP (i.e. TsConcPC and TsConcPE)	3.78	27.8	5.02×10^4	7.0	4.05	30.6	1.07×10^5	7.5
7.	Supernatant, 2S	-	-	2.66×10^5	37.1	-	-	5.65×10^5	39.8
8.	Concentrate supernatant 2S using UM 02 filters Concentrate = 2SConc (i.e. 2SConcC and 2SConcE)	4.50	33.0	2.31×10^5	32.3	4.48	35.00	5.20×10^5	36.6
9.	Filtrate = 2S Fil	-	-	2.79×10^4	3.9	-	-	4.51×10^4	3.2

pr = protein

See legends to Figures 26 and 27.

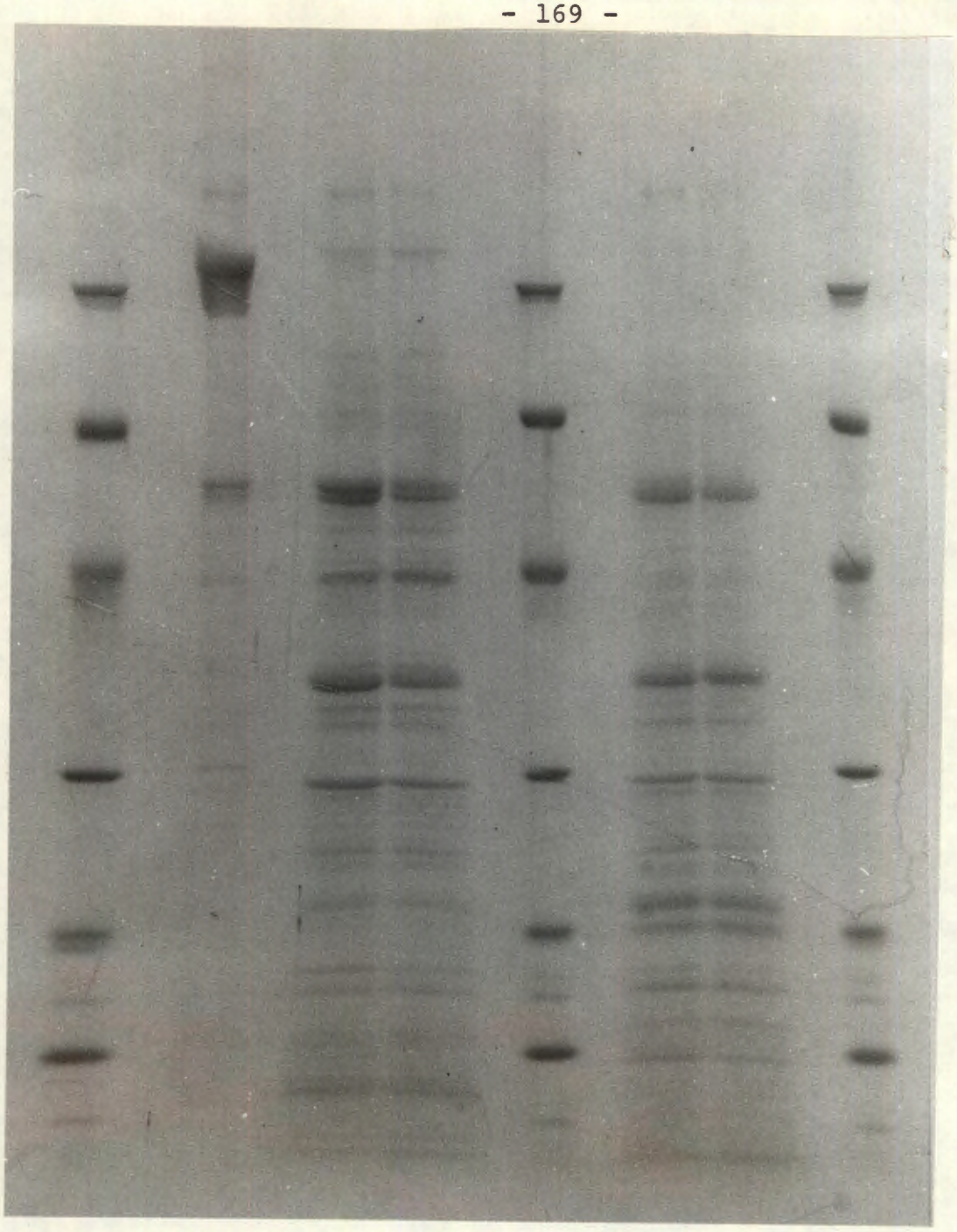


FIGURE 26 SDS-Polyacrylamide Slab Gel Electrophoresis of Peptide Material in the Pellet, T_p and Supernatant, Ts, of ^{14}C-NEM Modified Control and EGTA-Treated SR Vesicles Following Digestion with Trypsin

SR vesicles, 2.4 mg/ml (amount of protein = 30 mg), were incubated in 10 mM Tris-HCl, pH 7.4 and 5 mol NEM/mol ATPase for 90 min at 25°C. The vesicles were immediately taken to 37°C and EGTA (5 mM) and ^{14}C-NEM (33µM; 3 000 cpm/nmol) were added to the EGTA-treated vesicles. No EGTA was added to control vesicles.

After 5 mins excess β-mercaptoethanol (100 mM) was added and the reaction mixture was placed on ice. EGTA (5 mM) was added to control preparations. $CaCl_2$ (6.15 mM) and Tris-HCl, pH 9.8 (9.45 mM) were added to both preparations. The vesicles were washed twice by centrifugation at 110 000 × g for 30 min in 10 mM Tris-HCl, pH 7.4 and resuspended in the same buffer. The modified vesicles at a concentration of 1 mg/ml in 1 M sucrose were treated with 200:1 protein to trypsin for 15 min at 25°C (double cleavage). The vesicles were centrifuged at 110 000 × g for 30 min in 10 mM Tris-HCl, pH 7.4. The amount of protein and ^{14}C-NEM in the pellet, T_p, (insoluble fraction) and supernatant, T_s, (soluble fraction) were determined (Table 8). 25 µg of each fraction was layered onto a SDS-polyacrylamide slab gel according to the method of Laemelli (1970) as described in "Methods".

A = SR proteins

B = insoluble fraction of control vesicles, TpC

C = insoluble fraction of EGTA-treated vesicles, TpE

D = protein standards

E = soluble fraction of control vesicles, TsC

F = soluble fraction of EGTA-treated vesicles, TsE

vesicles. There were, however, differences in the tryptic fragment patterns in the pellet compared to the supernatant fractions. The pellet contained fragments of molecular weights 55 000, 45 000, 39 000, 28 000 and small amounts of lower molecular weight species. In the supernatant, however, there was only trace amounts of the 45 000 fragment and increased amounts of fragments of molecular weight approximately 20 000. Thorley-Lawson and Green (1975) and Stewart et al. (1976) have shown that the 45 000 dalton fragment contains a slightly higher proportion of non-polar amino acids (60% nonpolar and 40% polar amino acids) compared to the 55 000 dalton fragment which contains about equal amounts of polar and non-polar amino acids. Concentration of the supernatant caused these soluble peptides to precipitate and produce a pellet, (TsConcP), containing only small amounts of ^{14}C-label. The supernatant (2S), however, contained the bulk of the label and concentration of this fraction (2S Conc) again increased the turbidity of the solution indicating that the soluble fragments were rendered insoluble. SDS-polyacrylamide gel electrophoresis of these fractions (Figure 27) produced patterns which were similar in the control and EGTA-treated preparations. The increase in radioactive labelling in EGTA-treated vesicles compared with control vesicles, appeared to be present on a fragment of molecular weight of about 10 000 daltons (Figure 27). There was approximately twice the amount of label in EGTA-treated preparations compared to control preparations. It was thought probable that this fragment, showing increased labelling, arose as a result of hydrolysis

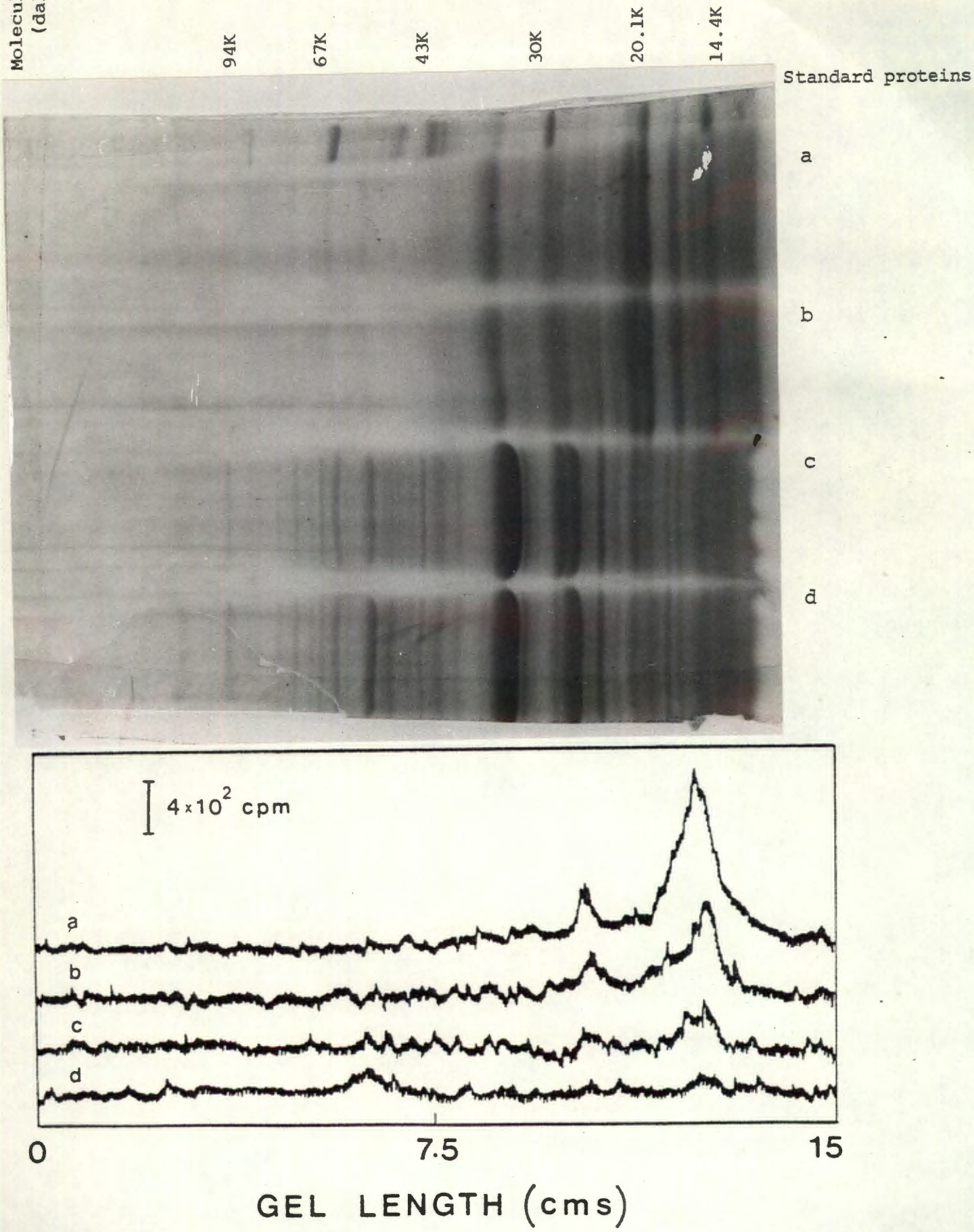


FIGURE 27

For details see following page.

FIGURE 27 SDS-Polyacrylamide Slab Gel Electrophoresis of the Peptide Material obtained by Concentration of the Supernatant, Ts.

SR vesicles were modified with NEM, EGTA-treated and digested with trypsin as described in Figure 26. The soluble fraction, Ts, (from control and EGTA-treated vesicles) was concentrated on an Amicon Ultrafiltration Apparatus model mmc using PM 10 filters. The concentrate is designated TsConc and the filtrate Ts Fil. The amount of radioactivity in both these fractions was determined (Table 8). The concentrated fraction TsConc was turbid, and a pellet formed following centrifugation at 230 000 × g for 30 min. The protein content and total radioactivity of the pellet, TsConcP, were determined as well as the total radioactivity in the supernatant, 2S (Table 8). The supernatant fraction, 2S, was concentrated using UM 02 filters. The concentrate, 2S Conc was analysed for protein content and total radioactivity. The filtrate, 2S Fil, was analysed for total radioactivity (Table 8). 250 µg of the fractions TsConcP (of control and EGTA-treated vesicles) and 2S Conc (of control and EGTA-treated vesicles) were layered on to SDS-polyacrymamide slab gels as described in "Methods". The radioactive material was detected by scanning the gel using a gas flow proportional counter fitted with a plotter.

a = 2S Conc E (fraction obtained from EGTA-treated vesicles)

b = 2S Conc C (fraction obtained from control vesicles)

c = Ts Conc PE (fraction obtained from EGTA-treated vesicles)

d = Ts Conc PC (fraction obtained from control vesicles)

of all the labelled fragments (i.e. 55 000 dalton, 45 000 dalton, 30 000 dalton and 20 000 dalton fragments). Very small amounts of ^{14}C - label were detected in gels of the soluble material which was precipitated following concentration (fraction TsConcP).

3.5.5.1 The Effect of High Speed Centrifugation, Temperature and β-mercaptoethanol on the Solubilization of SR Vesicles following Trypsinization

Solubilization of SR vesicles which had been subjected to various conditions (Figure 28) was monitored by the decrease in turbidity following addition of trypsin. High speed centrifugation enhanced the effect of a decrease in turbidity following trypsinization. This effect was increased by increasing the temperature to 37°C. β-Mercaptoethanol, however, appeared to have a protective effect against trypsinization as shown by a smaller change in absorbance relative to the other conditions. Figure 29 shows that in the presence of β-mercaptoethanol, the decrease in turbidity was less, following the addition of trypsin, both at 0°C and at 37°C.

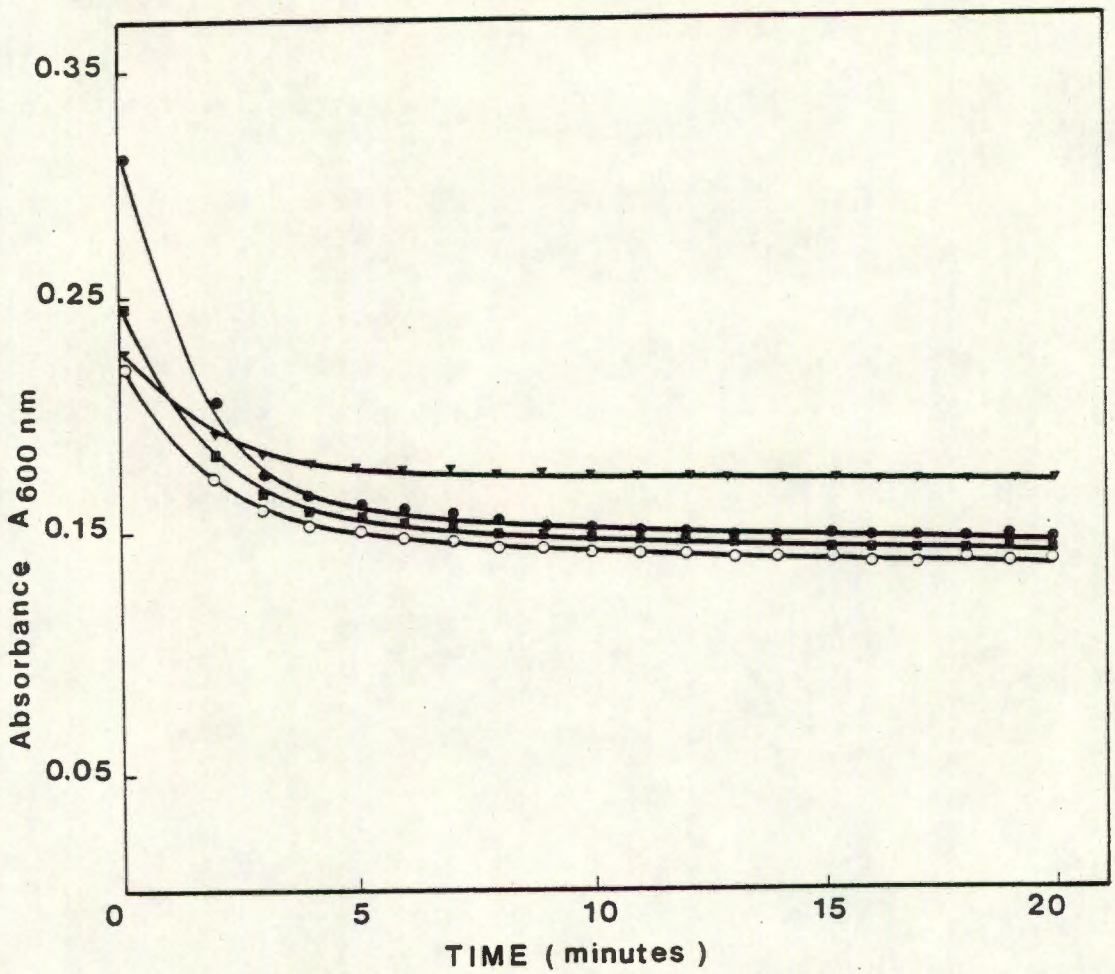


FIGURE 28 The Effect of High Speed Centrifugation, Temperature and β-Mercaptoethanol on the Solubilization of SR Vesicles Following Addition of Trypsin

SR vesicles, 2.4 mg/ml, in 10 mM Tris-HCl, pH 7.4 were subjected to the following procedures:

1. Incubated at 0°C for 5 min. (▼——▼)
2. Incubated at 0°C for 5 min. (■——■)
3. Incubated at 37°C for 5 min. (●——●)
4. Incubated at 37°C for 5 min thereafter β-mercaptoethanol (70 mM) was added (○——○).

Vesicles in preparations 2, 3 and 4 were centrifuged at 150 000 × g for 30 min and resuspended in 10 mM Tris-HCl, pH 7.4.

Each of the above preparations was made to a concentration of 1 mg/ml by the addition of sucrose to a final concentration of 1 M, and Tris-HCl pH 7.4 to 10 mM in separate 3 ml curvettes, which were placed in a Unicam spectrophotometer thermostatted at 25°C. Trypsin at a final concentration of 200.1 protein to trypsin was added and the decrease in turbidity was monitored at 600 nm for 20 mins. Graphs of time (mins) vs. absorbance were plotted.

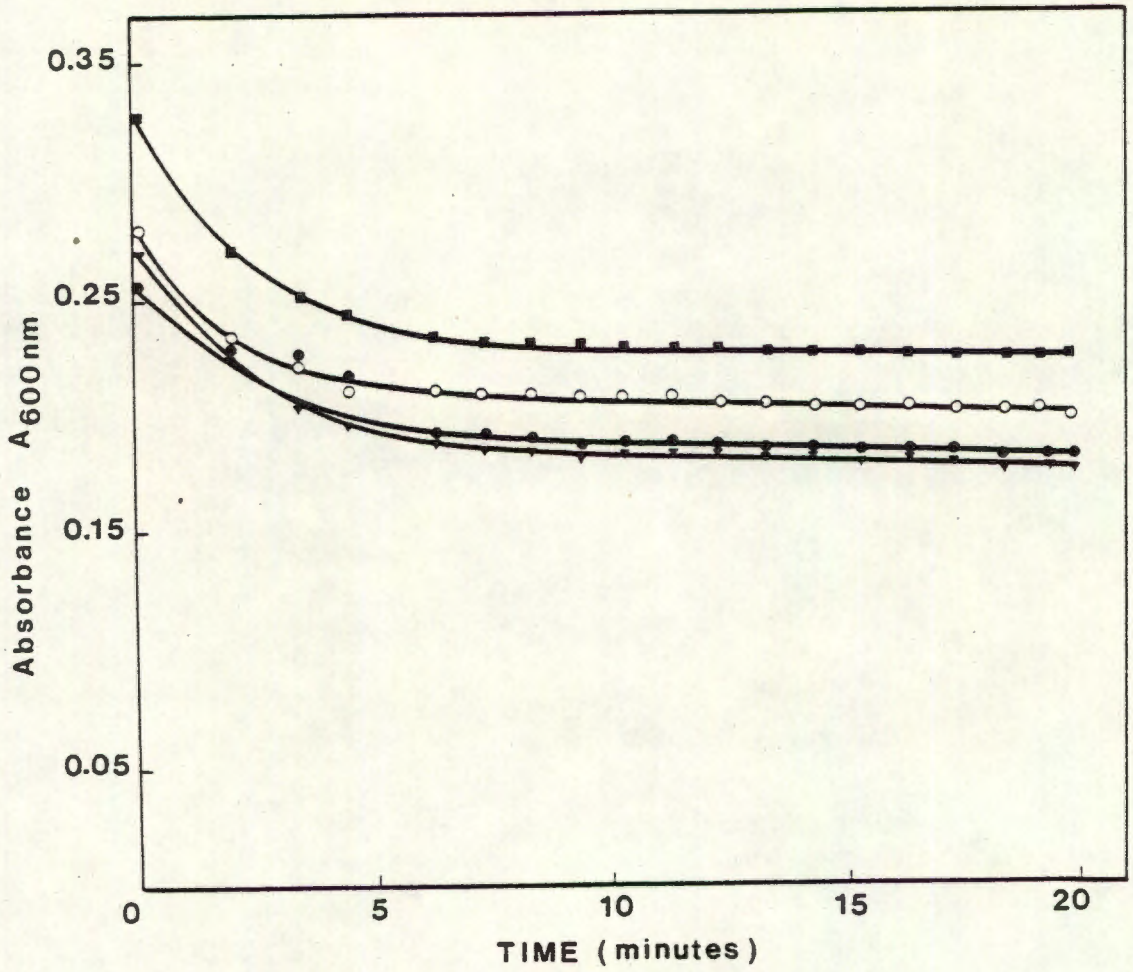


FIGURE 29 The Effect of Temperature and β-Mercaptoethanol on the Solubilization of SR Vesicles Following Trypsinization

SR vesicles, 2.4 mg/ml, in 10 mM Tris-HCl, pH 7.4 were incubated at 0°C for 5 min. (▼——▼) and 37°C for 5 mins. (■——■). Following incubation β-mercaptoethanol (70 mM) was added to a set of preparations that had been treated in the above manner, (●——●) 0°C + β-mercaptoethanol, (○——○) 37°C + β-mercaptoethanol. The preparations were centrifuged at 150 000 × g for 30 min., resuspended in 10 mM Tris-HCl, pH 7.4 and thereafter made to a concentration of 1 mg/ml in 3 ml curvettes by the addition of sucrose to a final concentration of 1 M and Tris-HCl, pH 7.4 to a final concentration of 10 mM. The curvettes were incubated at 25°C and trypsin added to a final concentration of 200:1 protein to trypsin. The decrease in turbidity was monitored at 600 nm for 20 min. The absorbance was recorded and plotted against time.

3.6 Peptide Mapping of NEM Modified (Ca^{2+}, Mg^{2+}) -ATPase of Control and EGTA-Uncoupled SR Vesicles

Peptide maps were prepared from NEM modified control and EGTA-treated SR vesicles, following extensive tryptic digestion. Prior to digestion, extrinsic proteins were extracted by treatment with DOC (10%;w/v). The lipid and higher molecular weight material was removed by passing the soluble peptide through a Sephadex G-100 column (Figure 30). The purified, digested ATPase, consisting of small peptides, which was shown to contain the bulk of radioactivity was freeze-dried and two-dimensional peptide maps were prepared according to the method of Michalak and MacLennan (1980) (Figures 31 and 33). The two-dimensional patterns were scanned for radioactivity by means of a gas flow proportional counter (Figures 32 and 34). Figure 35 shows a diagrammatic representation of the labelled peptides. The recovery of protein and radioactive label, which was monitored throughout the preparation, is reported in Table 9.

Peptide maps of the purified, trypsinized rabbit SR (Ca^{2+}, Mg^{2+})-ATPase of control and EGTA-treated vesicles corresponded to that described by Michalak and MacLennan (1980), who used rat SR vesicles and found 28 ninhydrin positive spots. Ninhydrin positive material in control and EGTA-treated two-dimensional peptide maps were identical; approximately 24 ninhydrin-stained spots could be identified in each case. Of these, 7 were uncharged and did not migrate on electro-

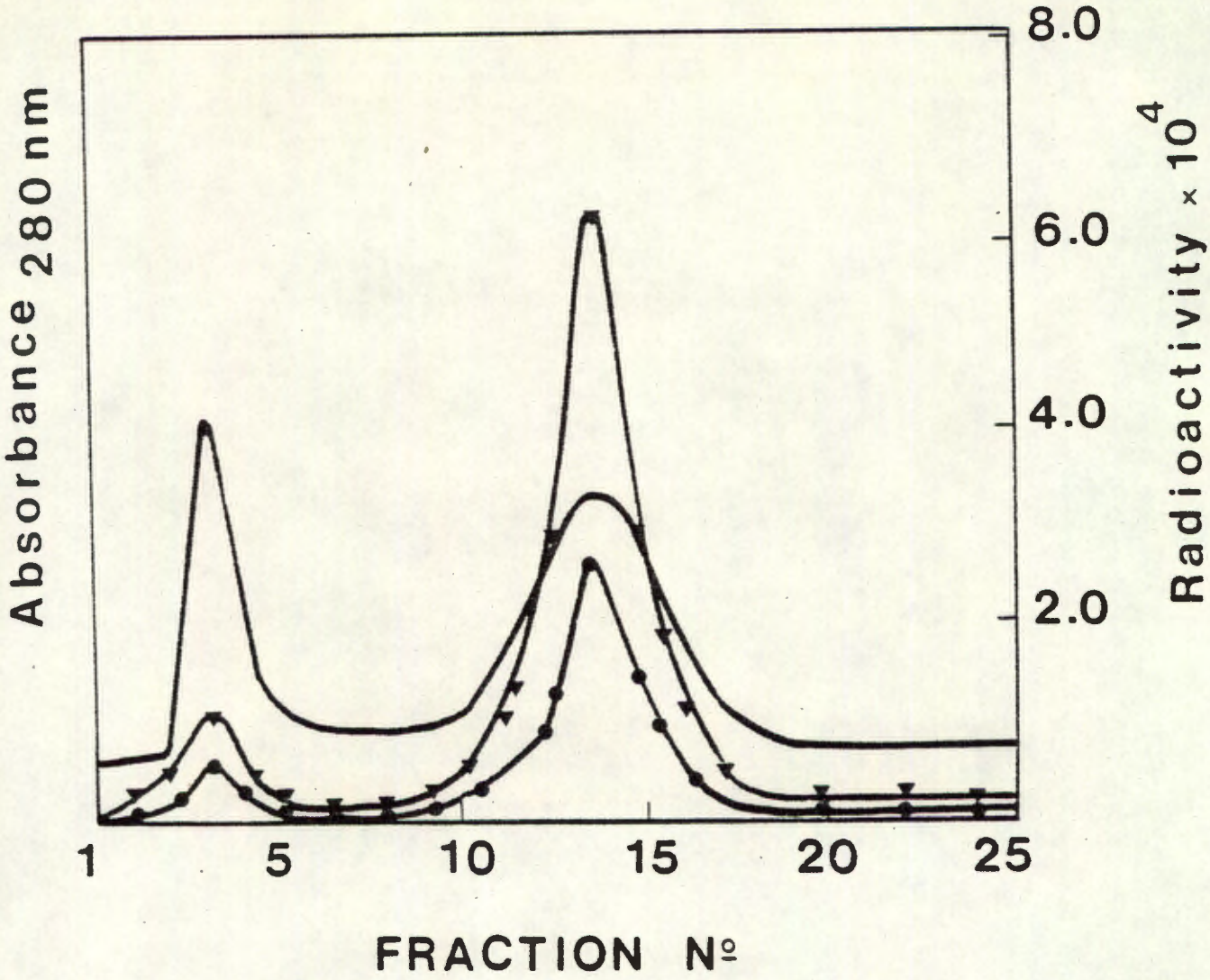


FIGURE 30 Elution Profile of Soluble Peptide Material, Obtained Following Extensive Tryptic Digestion, Passed Through a Sephadex G-100 Column

Control (●——●) and EGTA-treated (▼——▼) ^{14}C-NEM modified SR vesicles were extensively digested with trypsin as described in Fig.31. Following centrifugation at 150 000 × g, the soluble peptide material was passed through a Sephadex G-100 column (40 cm × 1.5 cm) and eluted with 1% NH_4HCO_3, pH 7.8. The eluant was passed through a Uvicord at 280 nm to measure protein absorbance (——). The high molecular weight fraction eluted in the void volume. Samples were collected at timed intervals and 25 µl were removed and the radioactivity in each sample assayed.

TABLE 9 Recovery of Protein and Radioactive Label Following NEM Modification Extraction of Extrinsic Proteins and Extensive Tryptic Digestion

Experimental Procedure	CONTROL				EGTA-TREATED			
	pr(mg)	% pr	cpm	% cpm	pr(mg)	% pr	cpm	% cpm
SR + ^{14}C-NEM	60.0	100.0	5.07×10^6	100.0	60.0	100.0	7.68×10^6	100.0
Pellet + ^{14}C-NEM + Spin	48.0	80.0	2.79×10^6	55.1	49.8	83.0	4.46×10^6	58.0
DOC TREATMENT Removal of Extrinsic Proteins								
SR + bound ^{14}C-NEM	48.0	100.0	2.79×10^6	100.0	48.0	100.0	4.30×10^6	100.0
SR + ^{14}C-NEM + DOC + 3 washes	32.2	67.0	2.25×10^6	80.6	33.6	70.0	3.65×10^6	84.9
TRYPSINIZATION								
ATPase + ^{14}C-NEM + TPCK trypsin	30.0	100.0	2.10×10^6	100.0	30.0	100.0	3.26×10^6	100.0
Pellet + ^{14}C-NEM	6.1	20.3	1.07×10^5	5.1	6.0	20.1	1.95×10^5	5.8
Supernatant + ^{14}C-NEM	-	-	1.97×10^6	93.8	-	-	3.02×10^6	92.6

pr = protein

For details see legends to Figures 31 and 33.

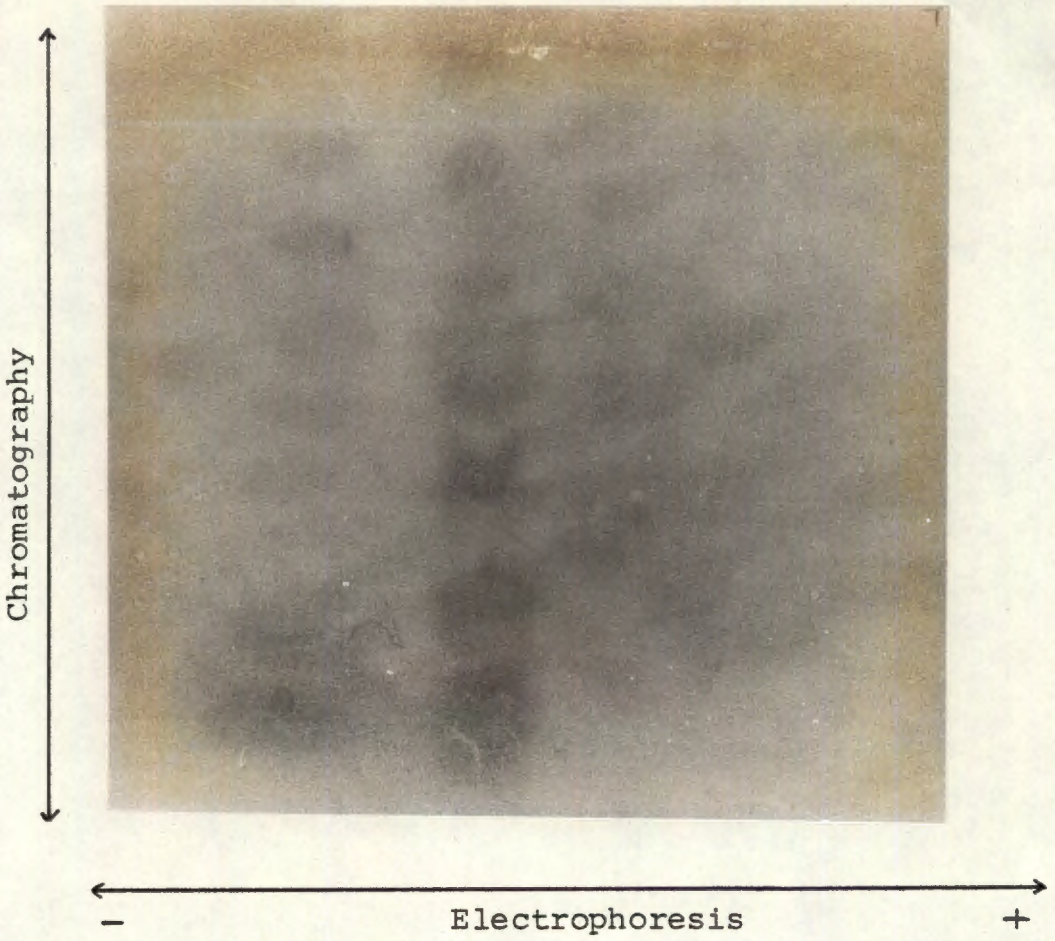


FIGURE 31 Peptide Map of Control, ^{14}C-NEM Modified (Ca^{2+}, Mg^{2+})-ATPase of SR Vesicles, Showing Ninhydrin Positive Peptides

SR vesicles, 2.4 mg/ml, (total volume = 25 mls) were incubated in 10 mM Tris-HCl, pH 7.4 and 5 mol NEM/mol ATPase for 90 min at 25°C. Thereafter the temperature of the reaction mixture was immediately brought to 37°C and ^{14}C-NEM (33 μM; 3000 cpm/nmole) in 10 mM Tris-HCl, pH 7.4 was added and the reaction was allowed to proceed for 5 mins. Excess β-mercaptoethanol (70 mM), EGTA (5 mM), were added. $CaCl_2$ (6 mM) was added in order to maintain the free calcium concentration at approximately 100 μM. The vesicles were centrifuged at 150 000 × g for 30 min and resuspended to a final concentration of 10 mg/ml in a solution containing 0.25 M sucrose and 10 mM Tris-HCl, pH 8.0. Removal of extrinsic proteins, extensive tryptic digestion, purification of soluble peptide material and peptide mapping were carried out as described in "Methods" (Section 2.3.2). Recovery of protein and radioactive label following NEM modification, extraction of extrinsic proteins and extensive tryptic digestion are shown in Table 9.

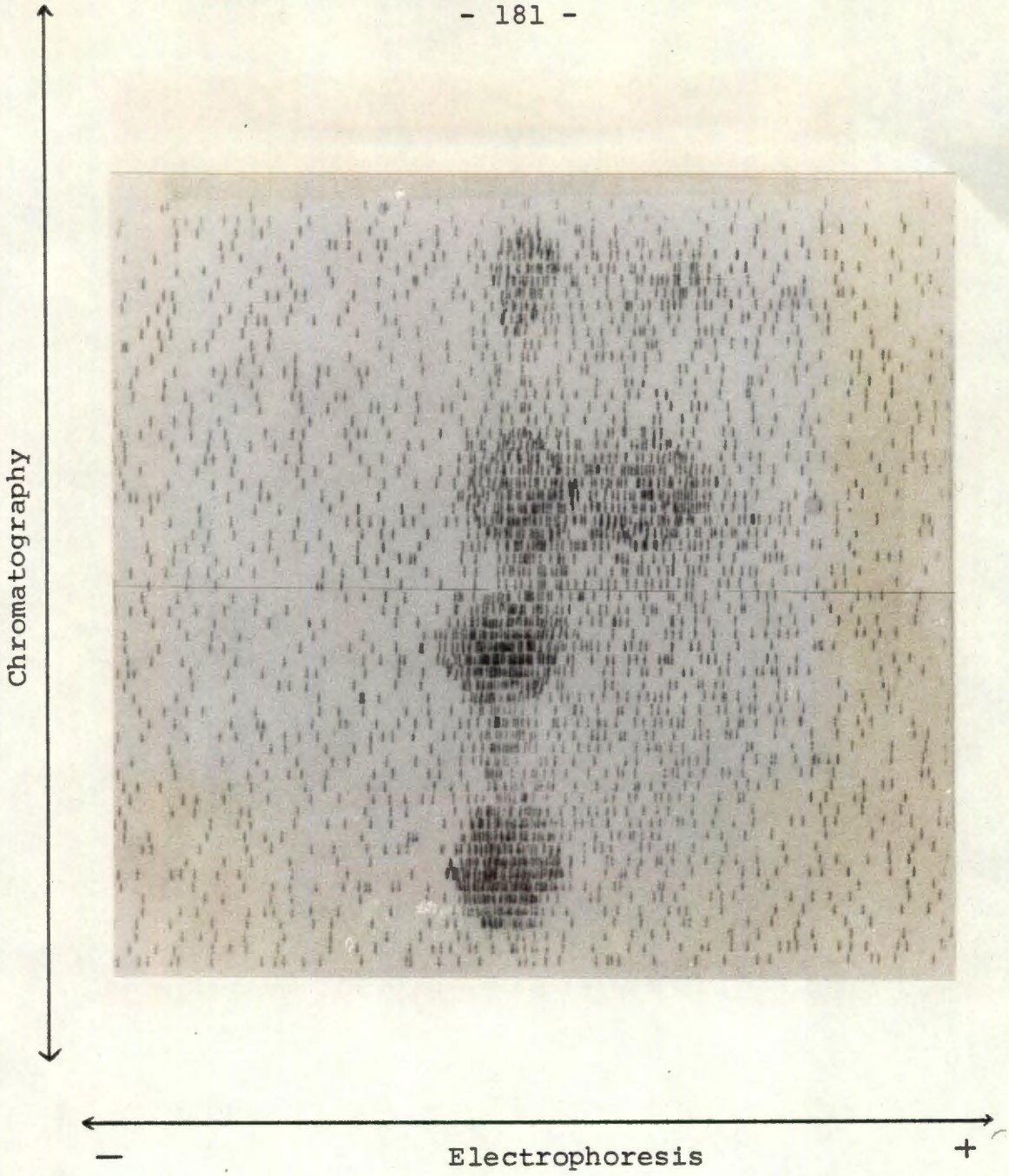


FIGURE 32 Detection of Radioactively Labelled Peptides on the Peptide Map of Control, ^{14}C-NEM Modified (Ca^{2+}, Mg^{2+})-ATPase of SR Vesicles

The radioactively labelled peptides on the peptide map of control (Ca^{2+}, Mg^{2+})-ATPase (Figure 31) were detected by scanning the peptide map using a gas flow proportional counter fitted with a plotter as described in "Methods" (Section 2.3.2).

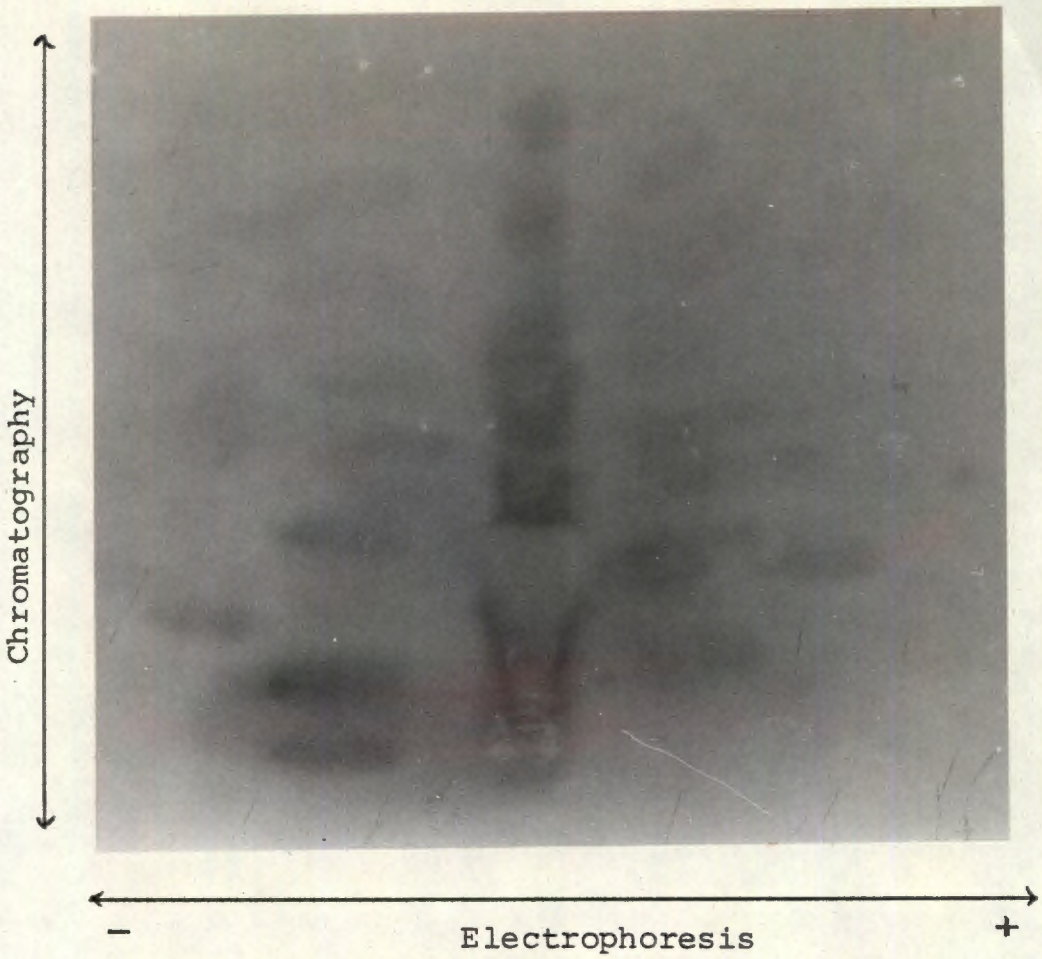


FIGURE 33 Peptide Map of EGTA-Treated, ^{14}C-NEM Modified (Ca^{2+}, Mg^{2+})-ATPase of SR Vesicles, Showing Ninhydrin Positive Peptides

SR vesicles, 2.4 mg/ml, were incubated in 10 mM Tris-HCl, pH 7.4 and 5 mol NEM/mol ATPase for 90 min at 25°C. The temperature of the reaction mixture was immediately brought to 37°C and EGTA (5 mM) containing ^{14}C-NEM (33 µM; 3000 cpm/n mole) was added and the reaction allowed to proceed for 5 mins. Excess β-mercaptoethanol (70 mM) and $CaCl_2$ (6 mM) were added. The vesicles were centrifuged at 150 000 × g for 30 min and resuspended to a final concentration of 10 mg/ml in a solution containing 0.25 M sucrose and 10 mM Tris-HCl, pH 8.0. Removal of extrinsic proteins, extensive tryptic digestion, purification of soluble peptide material and peptide mapping were carried out as described in "Methods" (Section 2.3.2). Recovery of protein and radioactive label following NEM modification, extraction of extrinsic protein and extensive tryptic digection are shown in Table 9.

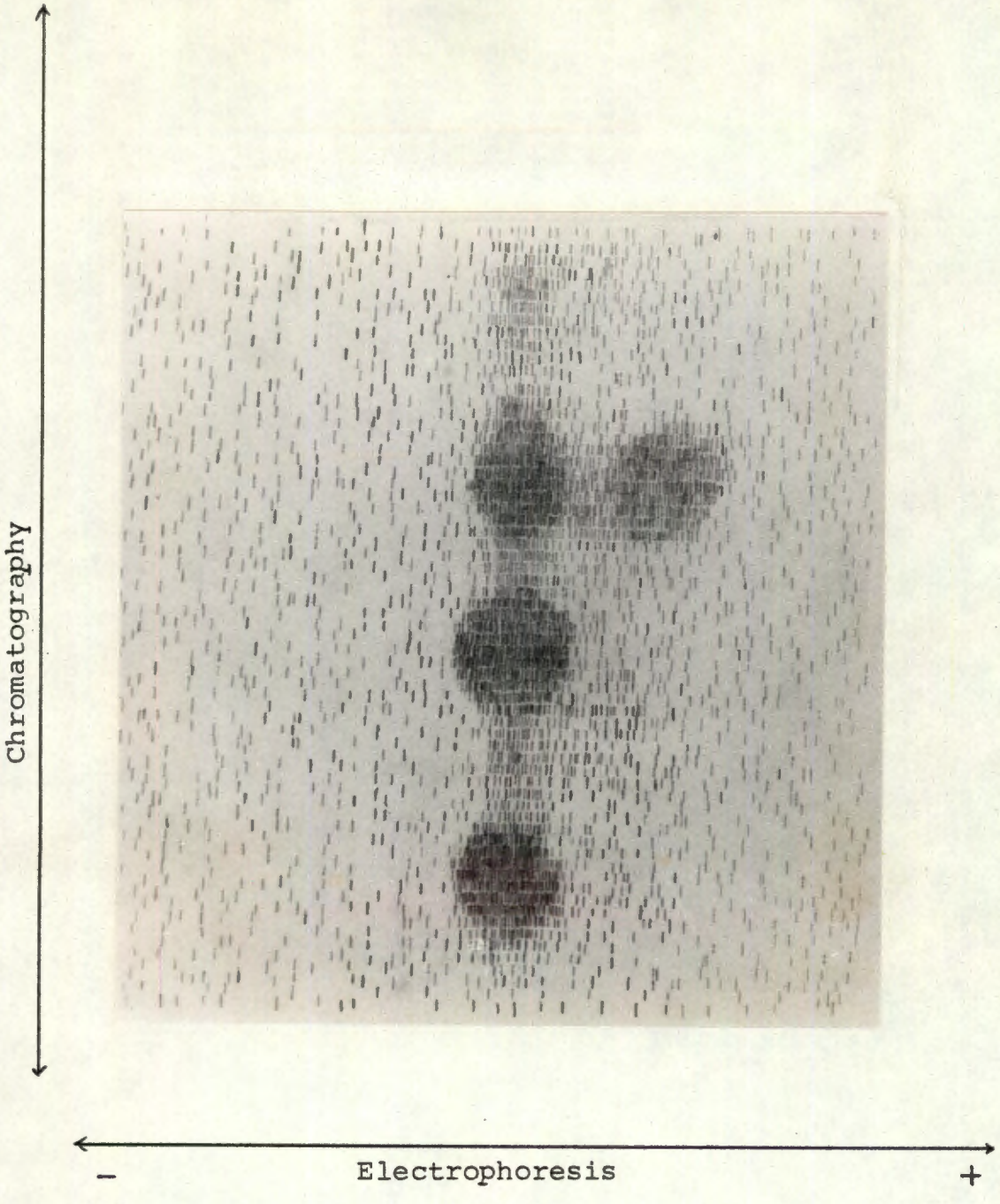


FIGURE 34 Detection of Radioactively Labelled Peptides on the Peptide Map of EGTA-Treated ^{14}C-NEM Modified (Ca^{2+}, Mg^{2+})-ATPase of SR Vesicles

The radioactively labelled peptides on the peptide map of EGTA-treated (Ca^{2+}, Mg^{2+})-ATPase (Figure 33) were detected by scanning the peptide map using a gas flow proportional counter fitted with a plotter as described in "Methods" (Section 2.3.2).

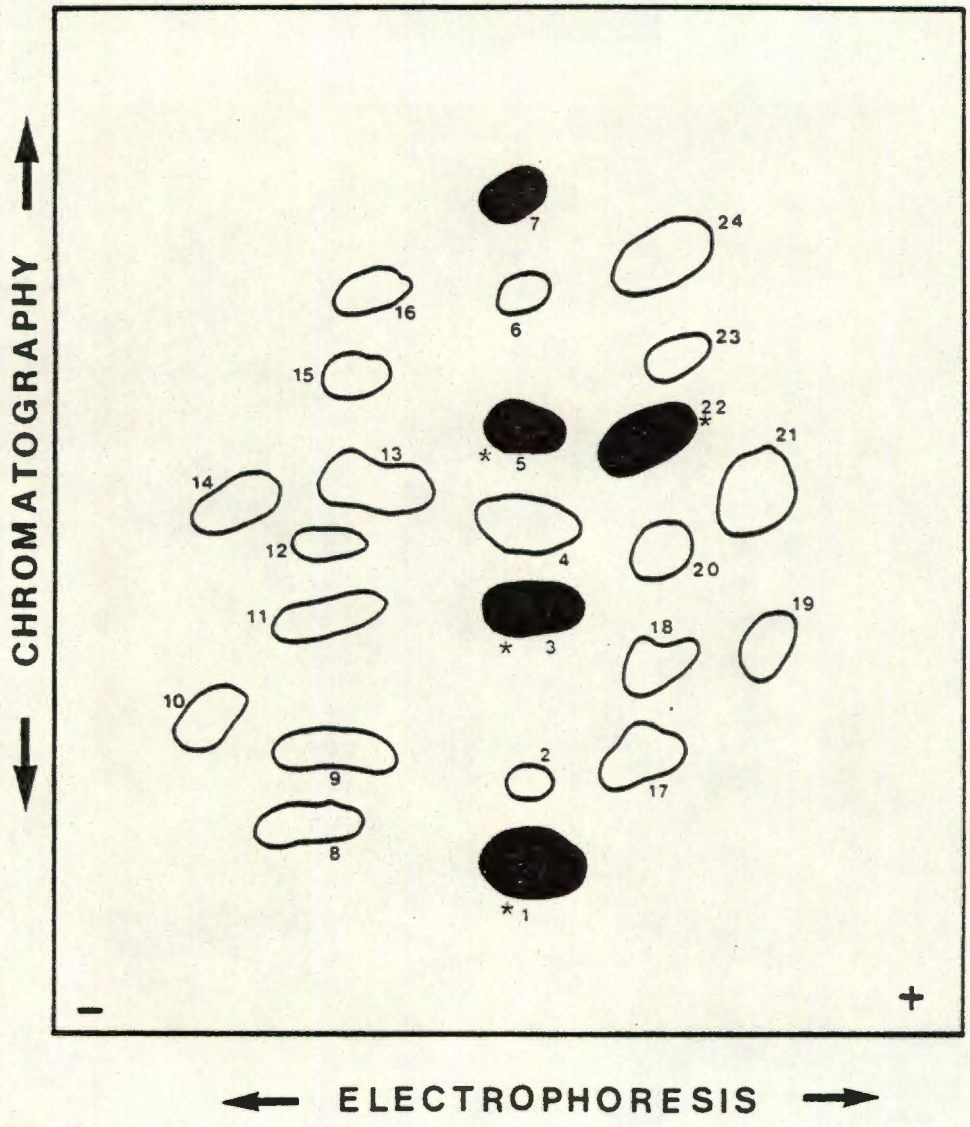


FIGURE 35 Diagram of the Correspondence Between the Peptide Patterns and the Radioactively Labelled Peptides

Ninhydrin stain of peptide patterns indicated by *empty circles*.

Radioactively labelled peptides indicated by *filled circles*.

*Increased ^{14}C-NEM labelling on peptides of EGTA-treated (Ca^{2+}, Mg^{2+})-ATPase compared to control peptides.

phoresis, 9 migrated towards the cathode and 8 migrated towards the anode. In both cases 5 peptides were radioactively labelled, 4 of which were uncharged and did not migrate on electrophoresis. The 5th radioactively labelled peptide migrated towards the anode. Radioactive label was increased on 4 peptides in the EGTA-treated vesicles, compared to control vesicles. Michalak and MacLennan (1980) examined,in their studies, protein labelled with [^{35}S]-methionine isolated from cells grown in rat skeletal muscle tissue culture. They compared the peptide map of the purified ATPase of the above labelled protein with that of the ATPase isolated from mature rat SR. They observed 28 ninhydrin-stained spots, 12 of which corresponded to the labelled peptides. The labelled peptides contained between 1 and 3 methionine residues, which corresponds to a total of 26 of a possible 32 methionines in the ATPase molecule. This is evidence that the peptides localized on the two-dimensional map do not overlap to a large extent.

4.0 DISCUSSION

4.1 Kinetic Reactivity of Thiol Groups of (Ca^{2+}, Mg^{2+})-ATPase

DTNB modification of native SR vesicles revealed four kinetic classes of -SH groups. A fast reacting class, moderately fast reacting class, slowly reacting class and unreactive class (Table 4). The total number of thiol groups estimated in the presence of SDS, using DTNB was found to be 20 -SH groups/1.5 × 10^5 g SR protein. Thorley-Lawson and Green (1977) showed that the purified ATPase of SR contained 20 thiol groups/115 000 daltons using DTNB or 1-^{14}C-NEM in SDS. Since our preparations of SR vesicles contain 85-90% ATPase protein (Fig.11), the value of the total number of thiol groups agrees favourably with those obtained by Thorley-Lawson and Green (1977). The kinetic reactivity of thiol groups of SR, using DTNB modification, was studied by Murphy (1976), Thorley-Lawson and Green (1977) and Andersen and Møller (1977). The conditions used by Murphy were identical to those used in this study, except that he used a manual mixing technique whereas the stopped flow method was employed in this study. Murphy analysed his results in terms of three classes of reactive -SH groups consisting of 2 fast, 8 moderate and 7 slow reacting -SH groups/mol ATPase with rate constants 8.3, 0.68 and 0.16 min^{-1}, respectively. The results in this study also showed 3 classes of reactive -SH groups. However, the reactivity of the fast class was an order of magnitude faster than that

reported by Murphy. This increased resolution was obtained by being able to monitor the first 5-10 seconds of the reaction by using the stopped flow apparatus. The moderately fast class in this study corresponded in reactivity and number to the fast class reported by Murphy, while the slowly reacting class represented the combined moderate and slow classes with rate constants similar to the 'slow' class reported by Murphy. Thorley-Lawson and Green (1977) found only two classes of reactive sites, with rate constants 33- and 1.9×10^{-3} sec^{-1}, respectively. The two classes were characterized, in a preparation of purified ATPase, by a ratio of rapidly reacting to slowly reacting sites of approximately 1:4, similar to that found in the study by Andersen and Møller (1977). The rate constants obtained by Thorley-Lawson and Green (1977) and Andersen and Møller (1977) are comparable when taking into account that the former authors performed their measurements at pH 8.4 and the latter authors at pH 7.5. However, the reactivity of the two fast reacting -SH groups in Murphy's study were approx. 40 times higher than those observed by the above workers and this could be due to the higher temperature (25°C) and higher DTNB concentration (4mM) used.

4.1.1 Effect of Acid and EGTA Inactivation on DTNB and NEM Modification of Thiol Groups

Treatment of SR membranes under mild acidic conditions or with EGTA resulted in an irreversible decline of calcium transport while ATPase activity was unimpaired (Fig. 4). This acid-induced uncoupling resulted in an increase in the number of fast and moderately fast reaching -SH groups, but a decrease in the total number of reactive thiol groups (Table 4). NEM modification of EGTA - and acid-treated vesicles showed similar patterns, i.e. an increase in the number of fast reacting -SH groups and decrease in the total number of reactive -SH groups (Figs.8 and 9). This increase in the number of fast reacting groups was probably due to a minor conformational change in the protein and partial unfolding of the polypeptide chain, resulting in a movement of the -SH groups from a restricted environment to a more accessible one in which the relatively bulky -SH reagents were sterically less hindered. There appears to be a shift in the reactivity of thiol groups, so that some thiol groups which were previously moderately fast reacting groups become fast reacting, some slowly reacting thiol groups become moderately fast reacting and some groups which were previously inaccessible now become slowly reacting as a result of a partial unfolding or minor perturbation of the protein (Table 4). This postulate is supported by the decrease in rate constants of the three classes observed with progressive inactivation of

transport. Moderately fast reacting groups which shift into the fast class have an effect of lowering the rate constants of the fast class (Table 4).

The decrease in total number of reactive thiol groups was due to autoxidation of the fast reacting -SH groups (Fig 10) and formation of intramolecular disulphide bonds thereby rendering the -SH groups inaccessible to the reagents. Hebdon et al. (1979) showed that oxidation of reactive thiol groups in SR, in the presence of rapidly reacting I_2, resulted in these groups readily forming intramolecular disulphide bonds. There was no evidence of crosslinking of ATPase monomers to yield higher molecular weight oligomers. In contrast, Murphy (1976a) showed the existence of higher molecular weight species following oxidation of thiol groups. However, this work was unable to be repeated by a number of investigators (Chyn and Martonosi, 1977; Louis et al. 1977; Hebdon et al., 1979) who only observed intramolecular disulphide bond formation. Extreme oxidizing conditions are required to bring about intermolecular crosslinking

Thorley-Lawson and Green (1977) observed that thiol groups disappeared during isolation of SR in the absence of dithiothreitol. This loss of thiol groups was not reversible and these groups could not be regenerated by reduction. The authors suggested that these thiol groups were oxidized to cysteic acid, since other partially oxidized intermediate would have regenerated cysteine when treated with dithiothreitol. Cysteic acid has been identified as an oxidation product of protein thiol groups in the study of the effects

of lipid hydroperoxides on proteins (Little and O'Brien, 1967). However, these authors suggested that it was unlikely that hydroperoxides formed from the highly unsaturated lipids of SR were responsible for the loss of thiol groups. They suggested that additional factors must be involved as there was little loss of thiol groups or subsequent storage of the ATPase at 4°C. The loss of reactive thiol groups in this study was probably due to intramolecular disulphide bond formation and not to cysteic acid formation as there was increased reactivity of approx. one -SH group/mol ATPase and the total number of reactive -SH groups decreased by approx. 2 mol/mol ATPase, implying that a disulphide bond was formed.

4.2 Quantitation and Localization of Thiol Groups Exposed during the Uncoupling Procedure

Uncoupling of SR membrane with EGTA in the presence of NEM to prevent autoxidation and crosslinking of thiol groups resulted in approximately one additional -SH group/ mol ATPase being labelled (Figs.12,14). The present results provide direct evidence that irreversible uncoupling of calcium transport from ATPase activity involves a conformational change of the ATPase polypeptide. Other evidence in support of this specific conformational change in the ATPase protein as a result of uncoupling, is loss of a tight nucleotide binding site (Aderem et al., 1979) and loss of a tight calcium binding site (Diamond et al., 1980). Stabilization of

of the transport system by calcium binding to high affinity calcium binding sites, with a $K_{dissociation}$ similar to the calcium binding site which activates catalytic activity, suggests that the conformational change is associated with the ATPase protein (McIntosh and Berman, 1978).

Polyacrylamide gel electrophoresis of the SDS-solubilized membranes showed that the additionally labelled -SH group was associated with the 100 000 dalton molecular weight (Ca^{2+}, Mg^{2+})- ATPase and not on the M_{55}, calsequestrin or glycoprotein (Fig.16). Hidalgo and Thomas (1977) found that fast reacting thiol groups were present in the minor glycoprotein component of SR vesicles. They suggested that the fast reacting -SH groups of SR were responsible for the "weakly" immobilized component seen on the EPR spectrum of SR labelled with maleimide spin label and that the major portion of this signal probably came from label bound to the glycoprotein. They further suggested that the observed spectral changes could not be an indication of the structural changes in the enzymes, as the spectra did not reflect exclusively the state of the (Ca^{2+}, Mg^{2+})- ATPase. The results in this study unequivocally showed that this fast reacting group exposed during uncoupling was located on the ATPase enzyme.

Mild tryptic digestion results in the cleavage of the 100 000 dalton ATPase into a 55 000 dalton catalytic fragment and a 45 000 dalton ionophoric fragment. Further cleavage of the 55 000 dalton fragment yields a 30 000 dalton polypeptide containing the phosphorylation site and a 20 000 dalton

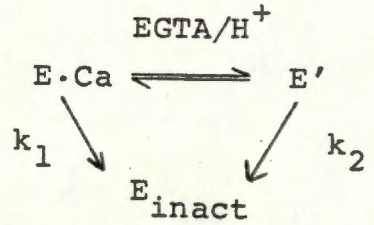
polypeptide which is thought to act as a Ca^{2+}-specific ionophore (Shamoo et al., 1976). Results of experiments attempting to localize the exposed -SH group on one of those subfragments were equivocal (Figs.18, 19). Although there was increased labelling on all the major tryptic subfragments of EGTA-treated vesicles compared to control, untreated vesicles, the major proportional increase in labelling appeared on the 55 000 dalton and 20 000 dalton subfragments (Table 5). There are several possible explanations for the variable and diverse labelling patterns.

(a) The lability of NEM and the possibility that it may react with side chain amino acids other than cysteine residues can be ruled out. At high concentrations of NEM and at alkaline pH (pH 8.0 and above), NEM reacts with the imadazole group of histidine and the α-amino group of amino acids and peptides (Smith et al., 1960, 1964; Brewer and Riehm, 1967). However, the concentrations of NEM ($\leq$ 1 mM) and pH range (7.0-7.4) used in this study are specific for the reaction of NEM with -SH groups. Similar conditions to the ones used in this investigation have been used by other workers to study the reaction of NEM with thiol groups of SR (Thorley-Lawson and Green, 1977; Hidalgo and Thomas, 1977; Yoshida and Tonomura, 1976). Disulphide exchange does not occur and thus cannot be used to explain the diversity of labelling.

(b) A further possible explanation for the random labelling of the tryptic subfragments is based on the concept of a dynamic state of the ATPase polypeptide within the lipid bilayer. The native associated (Ca^{2+}, Mg^{2+})- ATPase requires relative mobility within the lipid bilayer for maximal hydrolytic activity (Hidalgo et al., 1976). Several types of motions of proteins have been observed (Radda and Williams, 1976) (i) fast movement of many surface groups e.g. lysines (ii) slow flipping between conformations e.g. tryptophans (iii) internal breathing (iv) overall tumbling. Thomas and Hidalgo (1978) and Kirino et al. (1978) studied the rotational mobility of the (Ca^{2+}, Mg^{2+})- ATPase using saturation transfer EPR techniques and both detected the existence of rotational motion in the millisecond time range. More recently Bürkli and Cherry (1981) showed that this protein may undergo a different type of motion. They suggested that the ATPase is a flexible structure in which part of the molecule undergoes independent segmental motion. Such motion could be fast relative to vertical rotation of the whole protein in the membrane. They suggested that possibly all or part of the hydrophilic moeities, which protrude from the membrane, undergoes segmental motion.

Uncoupling of calcium transport from ATPase activity induced a conformational change in the protein resulting in exposure of an extra-SH group. This group was blocked with NEM, a procedure which immobilized the ATPase in a static and fixed conformation within the membrane. In addition to the conformational changes described above, the protein also undergoes minor fluctuations of conformation in the dynamic state (sometimes referred to as "breathing motion"). This motion is probably not identical in all the ATPase molecules and thus at a particular instant in time, the -SH group/mol ATPase which becomes exposed during uncoupling and which is anchored by NEM in a fixed conformation, is probably located in different positions on each ATPase molecule giving rise to the random labelling pattern observed.

(iii) Another possible explanation for the diversity of labelling could be due to the fact that the ATPase may exist in different conformational states during the process of uncoupling. The kinetics of inactivation of calcium transport and its dependence on calcium, suggest that the (Ca^{2+}, Mg^{2+})- ATPase can exist in two forms, a stable calcium form E·Ca and an unstable calcium-free form, E′, which are in rapid thermodynamic equilibrium as shown in the following scheme:



E′, which is induced by chelation of calcium or by the displacement of Ca^{2+} ions by protons from the calcium binding sites responsible for the stabilization of the ATPase, decays rapidly and irreversibly at 37°C to an inactivated state, E_{inact}, incapable of active transport, but which still retains calcium stimulated ATPase activity (Berman et al. 1977). E′ is more rapidly inactivated ($k_2 > k_1$) to the form E_{inact} than E·Ca.

In this presentation, where the inactivation of calcium transport is carried out in the presence of NEM in order to trap the exposed -SH group before autoxidation and intramolecular crosslinking occurred, there is no way of distinguishing between the two forms of the enzyme E′ and E_{inact}. Moreover, it is not possible to determine in which conformational state the enzyme exists after it is anchored in a particular conformation by NEM. Inactivation of the enzyme in the presence of NEM may not lead to the irreversible, E_{inact} state and the enzyme may be anchored in any intermediate state between $E \cdot Ca \rightleftharpoons E' \rightarrow E_{inact}$.

From the above discussion, however, it is conceivable that the enzyme may exist in a number of different dynamic conformations during the inactivation process. The existence of these different conformational states amongst the ATPase molecules nevertheless could give rise to the variable labelling patterns observed on the tryptic subfragments. This postulate is supported by the findings of Yamamoto and Tonomura (1977), who showed that in three different conformational states, i.e.

i.e. ^{Mg}E, $^{Mg}E_{ATP}$ and E ~ P, the number and localization of exposed lysine residues in each mole of tryptic subfragments, of molecular weights 50 000, 32 000 and 22 000, respectively, varied with the enzymic state. These authors also suggested that the possibility exists that the state of the ATPase in the membrane is somewhat heterogeneous. This conclusion was reached as the number of 2, 4, 6-trinitrobenzenesulphonate-modified lysine residues in each subfragment was not always an integral number, while their sum per mole of ATPase molecule usually was. Therefore it is possible that the diverse labelling patterns of the tryptic subfragments (Figs.18,19) and the peptide maps (Figs. 31,33) are a result of either the enzyme existing in different defined conformational states or due to heterogeneity within the ATPase populations. The peptide maps obtained were similar to those previously reported by Michalak and MacLennan (1980). They were able to account for 26 of a possible 32 methionine residues in the peptides of the ATPase molecule and this suggests that the peptides are non-overlapping. Of the total of 24 peptides found in this study, five were radioactively labelled in EGTA-treated and control preparations, four of these peptides showed increased labelling in the EGTA-uncoupled vesicles (Figs.32,34). In this case as well as with the tryptic cleavage products, labelling of approx. one -SH group/mol ATPase during uncoupling should produce an increase in labelling on one tryptic subfragment and one non-overlapping peptide. However, the fact that four peptides showed increased labelling in EGTA-treated preparations compared

with control preparations implies heterogeneity of the ATPase molecules.

It appears therefore, that an irreversible conformational change is responsible for the uncoupling of the catalytic domain from the ionophoric domain of the enzyme. These domains appear to be conformationally coupled and their functions are interrelated by the transmission of conformational energy which permits the hydrolysis of ATP to drive the vectorial transport of calcium across the membrane. This conformational energy may be transmitted through transmembrane parallel α-helical segments as in the purple membrane protein, bacteriorhodopsin from Halobacterium halobium whose known function is that of a light-driven proton pump (Henderson, 1977; Henderson and Unwin, 1975).

It is also possible that some types of conformational changes are precluded due to the fact that proteins exist in dynamic states and are oscillating between a number of these conformational states. Some of these states may be able to couple while others might not be conformationally correct and thus unable to couple ATPase activity to calcium translocation.

4.3 The Effect of NEM Modification on the Tryptic Digestion of SR Proteins

Centrifugation of tryptic digested (ratio of 1 000 protein to trypsin), NEM-labelled SR protein resulted in an increased concentration of soluble protein as well as an increase in radioactive NEM in the supernatant fraction. More extensive trypsinization (ratio of 200 protein to trypsin) enhanced this solubilization from 25% to 40% protein appearing in the supernatant (Table 6). As approximately 75% of the NEM-labelled -SH groups are released into the supernatant (Table 6), it is likely that they are situated on a hydrophilic, exposed domain of the protein. Yoshida and Tonomura (1976) found that trypsinization of NEM-modified SR proteins resulted in the solubilization of approximately 50% of the bound NEM. Trypsinization (ratio of 200 protein to trypsin) of control (unmodified by NEM) SR membranes resulted in the solubilization of 25% of protein (Table 7) in agreement with Inesi and Asai (1968) who found that tryptic digestion of SR vesicles caused a decrease in turbidity of SR membrane preparations and the loss of approximately 20% of the protein following centrifugation. There was a significant decrease in absorbance when SR membranes were trypsinized, the effect being more pronounced at 37°C than at 0°C (Fig. 28). β-Mercaptoethanol protected SR membranes against the decrease in absorbance induced by trypsin (Fig. 29).

NEM modification thus appeared to affect the extent of trypsinization, although the tryptic cleavage patterns and

the mobility of the major tryptic subfragments appear to be unaffected (Figs. 24,25). Yamamoto and Tonomura (1977) found that the tryptic digest of SR ATPase was unaffected by changing the concentrations of trinitrophenol which blocks lysine residues and also by changing the enzymatic state of the enzyme. Thorley-Lawson and Green (1977), when determining the distribution of thiol groups amongst the tryptic subfragments, digested the enzyme prior to adding the cleaved protein to NEM. This procedure may have been carried out to circumvent the problem of loss of protein bound-label following trypsinization which would lead to an inaccurate estimate of the number of thiol groups on the subfragments.

The soluble protein containing the bulk of the label and which was liberated following trypsinization and high speed centrifugation was almost devoid of the relatively hydrophobic 45 000 dalton fragment (Fig. 26), which has been shown to contain approx. 60% nonpolar and 40% polar amino acids and has been postulated to be buried in the membrane (Stewart et al., 1976). The insoluble fraction contained all four tryptic cleavage products, i.e. the 55 000, 45 000, 30 000 and 20 000 dalton fragments, whereas the soluble fraction contained the 55 000 dalton fragment and its cleavage products (Fig.26). The fact that tryptic cleavage of NEM-labelled SR resulted in liberation of the hydrophilic domain of the protein leaving the hydrophobic domain membrane bound may provide the basis of a method for separating out

hydrophilic and hydrophobic fragments. It appears that NEM-labelling alters the conformation of the tryptic fragments such that the interactions of the hydrophilic domain with the hydrophobic domain are diminished or the affinity of this domain for the membrane is decreased thereby allowing it to be released.

Concentration of the soluble fraction, rich in NEM modified protein resulted in a fraction containing subfragments with molecular weights varying between approx. 40 000 daltons and 10 000 daltons. The increased labelling in EGTA-uncoupled preparations compared with control-coupled preparations appeared on a subfragment of molecular weight approx. 10 000 daltons (Fig. 27). The most likely explanation is that this is an hydrolytic product derived from all the previously labelled tryptic fragments.

A working hypothesis based on this and previous studies suggests that the catalytically active ATP hydrolysing site is functionally and spatially distinct from the calcium translocating or ionophoric site on the ATPase protein. In the native enzyme these domains are conformationally coupled so that the "conformational" energy derived from ATP hydrolysis can be transmitted to the calcium translocating site. Acid- and EGTA-uncoupling of calcium transport from ATPase activity has now been shown to cause a minor perturbation or partial unfolding of the ATPase polypeptide, thus preventing conformational coupling between the two domains and the transmission of this "conformational" energy. The minor conformational change

is shown by increased reactivity of approx. one -SH group/ mol ATPase. Unfortunately, under the conditions used in this study it was not possible to label solely this -SH group. All experiments were performed by comparing the difference in labelling in uncoupled preparations to a control coupled vesicles. Further studies in this field, which will prove interesting, would be to "trap" the enzyme in a known conformation, e.g. E_{inact} and then look at the -SH reactivity. This can be done by inactivating the enzyme under anaerobic conditions, which would prevent autoxidation of -SH groups and allow the enzyme to proceed to the irreversible E_{inact} conformation. The -SH group reactivity in this state can then be compared with the -SH group reactivity of the enzyme in another known conformation, i.e. E.Ca. Also, once the enzyme is in a known conformation, it may be possible to identify the -SH group involved in uncoupling and to localize it on a particular domain of the enzyme and on one of the cysteine-containing peptides identified by Allen and Green (1978). As already stated, the -SH group is most probably present in a hydrophilic domain of the protein and will therefore be present in the portion of the ATPase molecule which has recently been sequenced by Allen et al. (1980b). Identification of this -SH group on one of the above sequences will give insight as to the position of this group in the primary structure of the enzyme and its location with respect to the phosphorylation and ionophoric sites.

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