MEMBRANE RECONSTITUTION STUDIES ON THE IRREVERSIBILITY OF INACTIVATION OF SARCOPLASMIC RETICULUM OF RABBIT SKELETAL MUSCLE.

by

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ABBREVIATIONS

ATP	Adenosine triphosphate
DOC	Deoxycholate
DOL	Dioleoyl lecithin
DOPC	Dioleoyl phosphatidylcholine
DOPE	Dioleoyl phosphatidylethanolamine
DTT	1,4 - Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol bis (β-aminoethyl ether) N,N'-tetra-acetic acid
EP	Phosphoenzyme
LDH	Lactate dehydrogenase
NADH	Nicotinamide-adenine dinucleotide
PEP	Phosphoenolpyruvate
Pi	Inorganic phosphate
PK	Pyruvate kinase
pNPP	p-Nitrophenylphosphate
SDS	Sodium dodecyl sulphate
SR	Sarcoplasmic reticulum
TCA	Trichloroacetic acid
TEMED	N, N, N', N' -tetramethylenediamine
TRIS	2 amino - 2 - hydroxymethyl - 1,3 - propandiol

A B S T R A C T

Mild acid treatment or incubation in the presence of Ethylene glycol bis (β -aminoethyl ether) - N,N' - tetraacetic acid inactivates calcium transport by sarcoplasmic reticulum membranes but does not inhibit calcium stimulated ATPase activity. This inactivation is apparently irreversible. The purpose of the present study was to determine whether lipid-protein interactions, imposed by the transmembrane nature of the (Ca^{2+},Mg^{2+}) - ATPase contributed towards the irreversible nature of the inactivation. This was determined by studying the possibility of reactivating calcium transport in acid-inactivated sarcoplasmic reticulum vesicles by means of membrane reconstitution studies. Calcium transport activity was reconstituted in control and acid-inactivated sarcoplasmic reticulum vesicles by deoxycholate solubilisation and subsequent slow dialysis at room temperature. Reconstituted control sarcoplasmic reticulum had an average specific activity of 0,38 µmol calcium transported /minute/mg of protein. Acid-inactivated sarcoplasmic reticulum vesicles, in which calcium transport had been inactivated to 0.2 µmol Calcium transported/minute/mg of protein (10% of the original transport activity) were studied by reconstitution methods. Following reconstitution the isolated, reformed vesicles regained up to 1,5-fold transport activity when compared with the original acid-inactivated vesicles, indicating that acid-inactivation was partially reversible.

Protein composition of reconstituted control and reconstituted acid-inactivated sarcoplasmic reticulum vesicles was studied by SDS-gel electrophoresis. Both preparations showed that the M55 protein was incorporated into reconstituted vesicles whereas there was a preferential loss of the M45 calcium binding protein (calsequestrin). The removal of deoxycholate into the dialysate was studied by means of (Carboxyl-C^{14}) -deoxycholate. The kinetics of removal indicate that approximately 0,15 mg DOC remained associated per mg of protein even after exhaustive dialysis. Calcium efflux from reconstituted vesicles was followed by release of calcium into Ethylene glycol bis (β- aminoethyl ether) -N, N' -tetraacetic acid following active uptake

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in the presence of precipitable phosphate anions. Calcium efflux was slower from reconstituted vesicles than from original sarcoplasmic reticulum. The ability of acid-inactivated sarcoplasmic reticulum to bind Ca^{2+} or adenine nucleotides tightly was investigated. The capacity to bind calcium tightly was decreased from 1.43 nmol Ca^{2+}/mg protein in control to 0,96nmol Ca^{2+}/mg protein in acid inactivated sarcoplasmic reticulum. Similarly, the capacity to bind adenine nucleotides tightly decreased from 0,20 mol nucleotides/mol ATPase in control vesicles to 0,07 mol nucleotides /mol ATPase in acid inactivated vesicles. Following reconstitution the capacity for tight binding of calcium and adenine nucleotides increased to 2,4 nmol Ca^{2+}/mg protein and 0,24 mol nucleotides/mol ATPase respectively indicating that the capacity to bind both calcium and adenine nucleotides tightly is closely related to transport activity but not to calcium dependent ATPase activity.

These studies indicate that the protein - lipid interaction restrains the acid-inactivated sarcoplasmic reticulum from returning to its native conformation. Release of these constraints by deoxycholate followed by its removal results in reversal of the conformational change to that of the coupled native sarcoplasmic reticulum membrane.

1.0. INTRODUCTION

INTRODUCTION

1.1. Brief overview and aim of the study

In muscle cells, the interactions of the contractile proteins with each other and with ATP are regulated by the concentration of free ionised calcium in the cytoplasm (Ebashi et al., 1969). The cytoplasmic concentration of free Ca^{2+}, in turn, is regulated by the sarcoplasmic reticulum (SR), a network of membranes which surrounds the muscle fibrils (Porter, 1961). When the muscle is stimulated, Ca^{2+} stored inside the sarcoplasmic reticulum is released into the cytoplasm, increasing the free Ca^{2+} concentration and producing contraction (Inesi, 1972). At the termination of the stimulus, Ca^{2+} is removed from the cytoplasm by an active transport mechanism and stored again within the SR. Hasselbach (1964) found the depletion of cytoplasmic Ca^{2+} by the sarcoplasmic reticulum to be Ca^{2+}, Mg^{2+} and ATP dependent and suggested that the (Ca^{2+} , Mg^{2+}) - ATPase (EC 3.16.13 ATP phosphorylase) is the active transport mechanism since ATPase activity and Ca^{2+} transport into the SR were related under various experimental conditions.

Active transport of calcium across the membranes of sarcoplasmic reticulum has received much attention for the past several years since this membrane system provides a simple and effective way of gaining an insight into the molecular basis of energy transduction (see Tada et al., 1978 for a review). Sarcoplasmic reticulum has been characterised with respect to its protein and lipid content (Section 1.2.) as well as its ability to store Ca^{2+} (Section 1.3.). The major protein of the sarcoplasmic reticulum, the (Ca^{2+} , Mg^{2+}) ATPase, constitutes 70 - 80% of total membrane protein and has been unequivocally established as the calcium pump through reconstitution experiments pioneered by Racker (1972) who first reported the formation of particulate liposomes from purified Ca - ATPase (Section 1.5.).

ATP hydrolysis in intact sarcoplasmic reticulum is coupled to calcium transport in a molar ratio of 1:2. Tight coupling

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of ATP hydrolysis and calcium transport depends on the integrity of the membrane and an apparent uncoupling can be induced by a number of agents which do not inhibit the ATPase activity but which cause increased passive permeability of SR vesicles to Ca^{2+} (Section 1.6.). These agents include diethyl ether (Inesi et al., 1967) phospholipase A (Feihn and Hasselbach, 1970) and EDTA at alkaline pH (Duggan and Martonosi, 1970). Evidence for proton-induced uncoupling of ATPase activity and calcium transport was presented by Berman et al.(1977), following brief exposure of sarcoplasmic reticulum membranes to mild acid conditions. The characteristics of the inactivation including stabilization via the high affinity calcium binding sites and large entropic and enthalpic contributions suggested a partial unfolding of the ATPase.

The reasons for this irreversibility are not obvious since there is no evidence for covalent modification of the ATPase. The aim of this study is to determine whether the restraints imposed by protein-lipid interactions contribute to an irreversible conformational change in the protein by means of reconstitutions experiments. Solubilization of the ATPase in detergent solutions, by abolishing protein-lipid interactions may allow the ATPase to resume its native conformation. This form of the ATPase should, when reconstituted with native sarcoplasmic reticulum lipids, be able to translocate Ca^{2+} ions, coupled to ATP hydrolysis.

The rest of the introduction reviews the literature and discusses the functional roles of the various components of the sarcoplasmic reticulum, the mechanism of Ca^{2+} accumulation and release by the sarcoplasmic reticulum, as well as a review of the methods of reconstitution, protein-lipid interactions and the effects of detergents on biological membranes. In Chapter 2, the different experimental procedures used in the course of this study are described. Chapter 3 presents the results obtained from the reconstitution experiments employed and these are discussed with reference to previous work in the field in Chapter 4.

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1.2. Structure and orientation of sarcoplasmic reticulum

The sarcotubular system of skeletal muscle closely surrounds the myofibrils and consists of two main parts, namely the transverse tubules (T-tubules) and sarcoplasmic reticulum (SR). The sarcoplasmic reticulum consists of a membranous network of continuous vesicles, tubules and cisternae which surround the myofibrils as well as forming enclosed compartments within muscle cells (Bennet and Porter, 1953; Porter and Pallade, 1957; Franzini - Armstrong et al., 1975.). The T-system is essentially invaginations of the sarcolemma having smaller dimensions than and never being continuous with the content of the sarcoplasmic reticulum (Franzini - Armstrong and Porter, 1964; Peachey, 1965). On both sides of each T-tubule, at the level of the A - I junction, two terminal cisternae are situated in close association with the T-tubules forming a triad. There is no direct communication between the different parts of the triad. The T-tubule and terminal cisternae are separated by a gap of 120 - 140Å (Franzini - Armstrong, 1970).

Podolsky and Constantin (1964) provided evidence that the release of intracellular Ca^{2+} and the release of Ca^{2+} at the level of the triad (i.e. the A - I junction) is essential for muscle contraction. It is generally accepted that the inward spread of the action potential along the T-tubule results in the release of Ca^{2+} from the sarcoplasmic reticulum at the level of the triadic junction. Calcium diffuses from the sarcoplasmic reticulum to the contractile proteins of the myofibril resulting in muscle contraction (see Fuchs, 1974 for a review on the possible mechanisms involved in excitation - contraction coupling).

1.2.1. Protein composition of SR membrane

Ostwald and Mac Lennan (1974) have isolated seven proteins from the sarcoplasmic reticulum. This raises doubts as to the purity of their SR preparation since studies on highly purified preparations show that only three protein components are present

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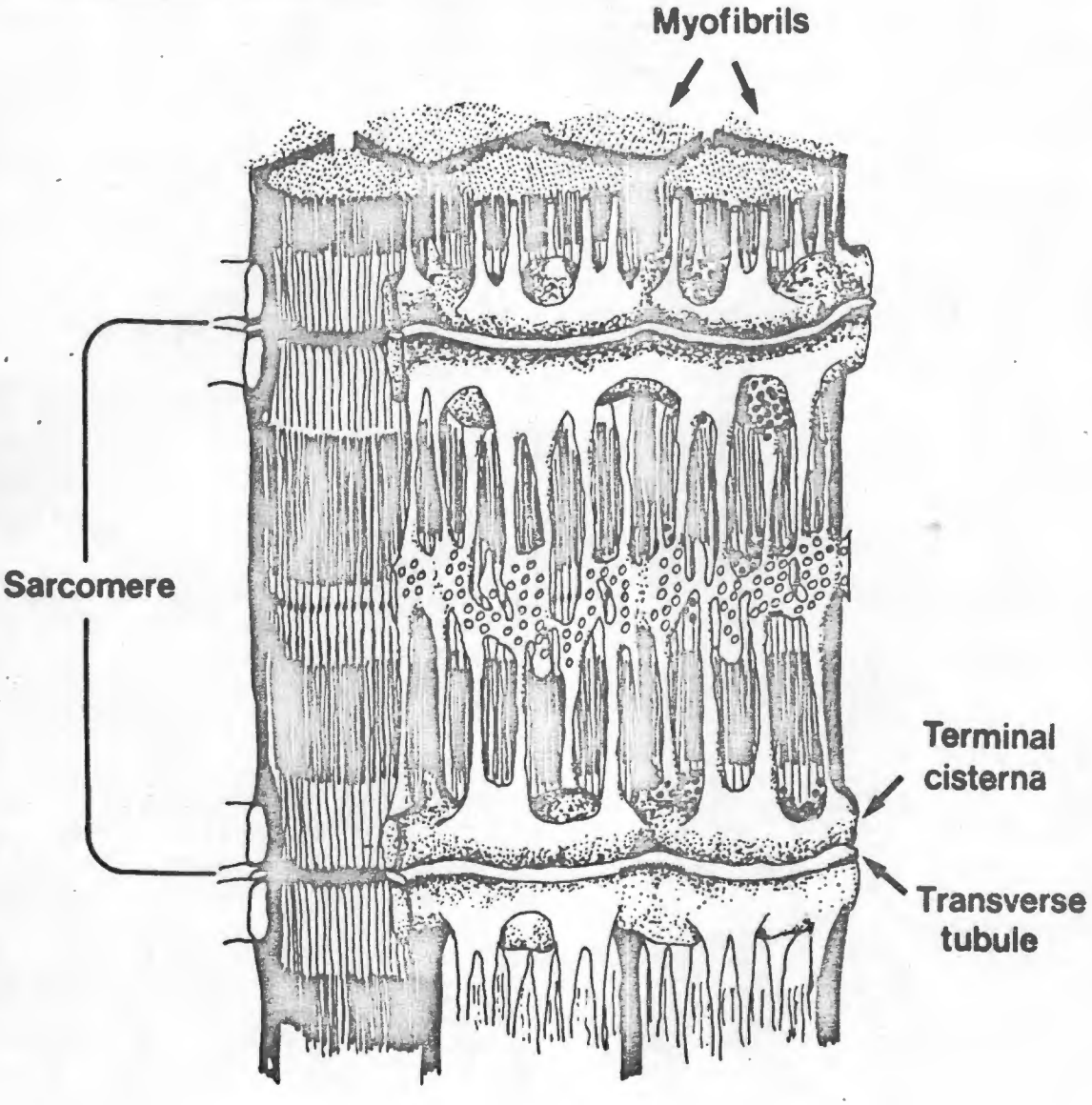

Plate 1. Schematic representation of the sarcotubular system of mammalian skeletal muscle (Redrawn from Peachey, 1965).

The transverse or T-tubules are invaginations of the sarcolemma and are in close association with the terminal cisternae of the sarcoplasmic reticulum forming triadic junctions at the union of the A and I bands.

in any significant amounts (Meissner et al., 1973; Meissner, 1974; Inesi and Scales, 1974; Eletr and Inesi, 1972). These three proteins are the ATPase protein (Mac Lennan, 1970; Inesi, 1972) calcium - binding protein and calsequestrin (Mac Lennan and Wong, 1971; Ostwald and Mac Lennan, 1974). The proteins of the sarcoplasmic reticulum can be classified as either extrinsic and intrinsic membrane proteins. The calcium binding proteins and calsequestrin constitute the extrinsic group which are loosely associated membrane proteins and 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).

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

The proteins of the sarcoplasmic reticulum were first separated successfully on SDS - polyacrylamide gel electrophoresis by Martonosi (1969) and Martonosi and Halpin (1971). Without correlative data from purified proteins it was impossible to state unequivocally that all the proteins were of sarcoplasmic reticular origin but three proteins of mol. wts 100 000, 60 000 and 50 000 constituting about 50% of the total protein were clearly of major content. The 100 000 mol wt protein was deduced to be the ATPase since ^{32}P from $[\gamma - ^{32}P]$ ATP was incorporated exclusively into this component (Martonosi and Halpin, 1971).

Mac Lennan (1970) first succeeded in isolating the ATPase enzyme with a two-fold increase in its activity. The enzyme was isolated as a phospholipid - protein complex containing small amounts of low molecular weight proteolipid following solubilization with sodium deoxycholate and subsequent ammonium acetate precipitation. Less perturbing detergents like cholate, lysolecithin and Triton X100 have subsequently been used to purify the ATPase (Peterson et al., 1978; Ikemoto et al., 1971;

Meissner/...

Meissner and Fleisher 1971; Le Maire et al., 1976).

Deamer and Baskin (1969) have used electron microscopy to investigate the concave faces of freeze fractured SR vesicles. They found 90 Å particles which Tillack et al.(1974) later claimed to appear in parallel with ATPase activity in the SR of developing muscle. Furthermore, while freeze-fracture faces of lipid vesicles are smooth, 90 Å particles are noted in vesicles reconstituted from lipids and purified SR ATPase (Packer et al., 1974). It is thus apparent that the 90 Å particles correspond to the ATPase protein (Scales and Inesi, 1976).

A further ultrastructural detail of sarcoplasmic reticulum vesicles was noted by electron microscopy on negatively stained preparations, which revealed the presence of 35 Å projections on the outer surfaces of the vesicles (Ikemoto et al., 1968; Inesi and Asai, 1969). The outer projections,like the 90 Å particles, appear in parallel with ATPase activity in the SR of developing muscle (Sarzala et al., 1975). Hardwicke and Green (1974) showed the presence of 35 Å projections in vesicles reconstituted with purified SR ATPase. These projections disappear after digestion with trypsin under conditions in which extensive cleavage of the ATPase protein may be demonstrated (Stewart and Mac Lennan, 1974; Inesi and Scales, 1974). Inesi and Scales (1974) showed that the removal of the minor protein components of SR vesicles (i.e. calsequestrin and calcium binding protein) by dialysis against EDTA did not result in the removal of either the 90 Å or the 35 Å particles, suggesting that they are both related to the ATPase protein. Deamer and Baskin (1969) observed a discrepancy between the number of 90 Å particles and the more numerous 35 Å projections. This was later confirmed by Scales and Inesi (1976) who found 3 to 4 times more 35 Å projections than 90 Å particles. They suggested that the discrepancy is due to oligomer formation by the hydrophobic ends of the molecules in the membrane while the hydrophillic, polar regions on the surface of the membrane, remained distinct (Scales and Inesi, 1976). While the discrepancy could also be ascribed to variable

penetration/...

penetration into the membrane of the polypeptide chain of the ATPase, the suggestion is supported firstly by evidence that the fully active, detergent - solubilised ATPase has a minimal molecular weight of approximately 40 000, corresponding to a trimer or tetramer (Le Maire et al., 1976) and secondly by the cross-linking data of Murphy (1976) who observed species of mol. wt 4×10^5 on SDS polyarylamide gels after cupric-phenanthroline cross-linking. In addition, Murphy observed no change in the electrophoretic mobilities of the calcium binding protein and calsequestrin as well as an absence of species relating to dimeric, trimeric or pentameric forms of the ATPase (Murphy, 1976).

1.2.1.2. Calsequestrin or M45-protein

Calsequestrin was first isolated and characterised by Mac Lennan and Wong (1971). The molecular weight of calsequestrin is estimated at between 44 000 and 55 000 (Mac Lennan and Wong, 1971; Meissner et al., 1973; Inesi and Scales, 1974; Margreth et al., 1974). Calsequestrin is an acidic protein with a high capacity for binding Ca^{2+} and it constitutes 5 - 10% of the total SR protein (Mac Lennan and Wong, 1971 ; Meissner et al., 1973). In the presence of 100mM KCl calsequestrin has a high capacity for binding Ca^{2+} (850 nmoles Ca^{2+}/mg) and low affinity. The dissociation constant is approximately 800 µM (Ostwald and Mac Lennan, 1974). 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 (Mac Lennan and Wong, 1971; Jilka et al., 1975; Garcia et al., 1975). In the presence of sarcoplasmic Ca^{2+} concentrations of less than 1 µM, calsequestrin would not be expected to bind a significant amount of Ca^{2+}. On the other hand the interior of the sarcoplasmic reticulum may contain Ca^{2+} concentrations of 10 - 20 mM (Mac Lennan and Wong, 1971.; Sandow, 1970). If all intra sarcoplasmic reticular calcium was in the ionised form it would be more than sufficient to saturate the calcium binding site of calsequestrin (Mac Lennan and Holland, 1975).

The location of calsequestrin with respect to the interior

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or exterior surface of the membrane is in doubt. Extraction data (Mac Lennan and Wong, 1971) indicate that the protein is not on the external surface of the membrane. In intact vesicles calsequestrin is not readily cleaved by trypsin; degradation by trypsin only being observed in later stages of the digestive process (Stewart and Mac Lennan, 1974). In addition, antibodies raised against calsequestrin, fail to aggregate sarcoplasmic reticular membranes arguing against an exterior location of calsequestrin (Mac Lennan and Holland, 1975).

In opposition to the idea of an internal location for calsequestrin, Duggan and Martonosi (1970) provided evidence for the extraction of calsequestrin with EDTA. While it could be argued that this provides evidence for an external location of calsequestrin it must be borne in mind that the EDTA extraction made the vesicles permeable to inulin and it is therefore probable that the experiment does not bear on localisation. Lactoperoxidase labelling of sarcoplasmic reticulum failed to throw any light on the location of calsequestrin since universal labelling of the proteins occurred (Mac Lennan et al., 1972; Thorley - Lawson and Green, 1973).

In summary the localization of calsequestrin is uncertain but the weight of evidence is in favour of internal localization.

1.2.1.3. Ca^{2+} -binding or M55 protein

A second acidic protein which binds Ca^{2+} was first isolated by Mac Lennan et al. (1972). This protein has a molecular weight of 55 000 dalton and binds Ca^{2+} with a high affinity but low capacity (16-22n moles/mg) with a dissociation constant of between 2,5 and 4,0 µM in the presence of 0,1M KCl (Ostwald and Mac Lennan, 1974). The localisation of the high affinity Ca^{2+} - binding protein seems to be identical to that of calsequestrin. Although calsequestrin is more readily extracted with EDTA than is the high affinity Ca^{2+} binding protein (Duggan and Martonosi, 1970), both are ultimately extractable with EDTA.

Lactoperoxidase/ ...

Lactoperoxidase - catalysed iodination, tryptic digestion and antibody tagging, suggest a similar localization of calsequestrin and high affinity Ca^{2+} binding protein (Mac Lennan and Holland, 1975). It seems likely that their functions are related and it has been speculated by Jilka et al.(1975) that the two minor SR proteins may play a role in regulating membrane permeability.

1.2.2. Lipid composition

Phospholipids comprise about 80% of the total lipid on a molar basis. The remainder consists of neutral lipid of which 95% is cholesterol. Phospholipids of sarcoplasmic reticulum consists of phosphatidylcholine (65 - 73%), phosphatidylethanolamine (12 - 19%), phosphatidylinositol (about 9%), phosphatidylserine (about 2%), sphingomyelin (about 4 %) and cardiolipin (0,1 - 0,3%) (Martonosi et al., 1968; Meissner and Fleischer, 1971; Waku et al., 1971; Owens et al., 1972). Although phosphatidylcholine alone was sufficient for the activation of ATPase activity, and phosphatidylethanolamine was sufficient for the reconstitution of calcium transport activity (Knowles et al., 1976; see section 1.5 and 1.6.) it is not understood how the diversity of the phospholipids found in the native membrane account for the Ca^{2+} transport function.Of the approximately 90 lipid molecules per molecule of ATPase in native sarcoplasmic reticulum, about 30 are found in close association with the protein (see section 1.5 and 1.6).

1.2.3. Proteolipid

Mac Lennan et al.(1972) isolated a proteolipid from sarcoplasmic reticulum by sequential extraction with organic solvents. SDS polyacrylamide gel electrophoretic analysis showed the proteolipid to constitute a few percent of the total protein

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and indicated a molecular weight of 6000. Aminoacid analysis, however, indicates a molecular weight of 12000. Racker and Eytan (1975) found that a heat-stable factor, resembling the proteolipid increased the coupling ratio for transport in particulate liposomes reconstituted from purified Ca - ATPase and exogenous phospholipids. The function of the proteolipid has not been resolved.

1.2.4. Proteolytic fragmentation of the ATPase

The hydrophillic region of the ATPase (MW 100 000) is exposed to the exterior of the membrane while its hydrophobic region interacts directly with boundary phospholipids (Stewart et al., 1976). Brief exposure of sarcoplasmic reticulum to trypsin in the presence of IM sucrose causes the ATPase protein to be degraded into two fragments of molecular weight 55 000 and 45 000 respectively (Inesi and Scales, 1974; Stewart and Mac Lennan, 1974; Thorley - Lawson and Green, 1973). The larger fragment (MW 55000) is phosphorylated by $[\gamma - {}^{32}P]$-ATP, indicating the presence of the phosphorylation site on this fragment (Thorley-Lawson and Green, 1973). The 45000 molecular weight fragment appears more hydrophobic than the 55 000 molecular weight fragment, having a larger percentage of nonpolar amino acids residues (Stewart et al., 1976). This is in agreement with earlier work by Stewart and Mac Lennan, (1974) who used antibodies to study the arrangement of the tryptic fragments in the membrane. They found that the 55000 dalton fragment was more accessible to antibody than the 45000 dalton fragment, suggesting that the former is more exposed to the surface of the membrane while the latter is inaccessible (Stewart and Mac Lennan, 1974).

Extensive tryptic digestion of the 55000 dalton fragment yields subfragments of 30 000 and 20 000 daltons (Thorley - Lawson and Green, 1973; Stewart et al., 1976; Mac Lennan et al., 1971).

Labelling/....

Labelling of the active site with [γ -^{32} P]-ATP or [^{3}H]-N-ethyl - maleimide, prior to proteolytic cleavage, results in the incorporation of both labels into the 55 000 and 30 000 dalton fragments, indicating that these contain the active site. Antigenic cross-reaction studies indicate that the 20 000 dalton subfragment is also derived from the 55 000 dalton fragment (Stewart et al., 1976). Black lipid membrane conductance assays have shown the 100 000 dalton ATPase, the 55 000 dalton fragment and the 20 000 dalton subfragment to exhibit ionophoric activity (Shamoo and Mac Lennan, 1974; Thorley-Lawson and Green, 1973; 1975; Shamoo et al., 1977 a,b). The 20 000 dalton subfragment specifically requires Ca^{2+} ions for expression of its ionophoric properties and has a selectivity sequence of divalent cations consistent with the selectivity of transport in intact sarcoplasmic reticulum (Shamoo et al., 1977 a). In addition, ruthenium red and mercuric chloride, inhibitors of transport in the intact system, inhibit the ionophoric activity of the 55000 and 20000 dalton fragments while methylmercury, an inhibitor of the ATPase site, does not inhibit ionophoric activity (Shamoo and Mac Lennan, 1974). Shamoo et al., (1977 a) have postulated that the (Ca^{2+} , Mg^{2+}) - ATPase consists of a hydrophobic channel (45 000 fragment) spanning the membrane and that the hydrophilic portion (55 000 fragment) on the exterior cytoplasmic surfaces contains both the 30 000 dalton phosphorylation site and 20 000 dalton ionophoric site. They further postulated that the 20 000 dalton subfragment acts as a 'gate' partially buried in the hydrophobic interior at the head of the 45 000 dalton fragment.

1.3. Calcium accumulation and release by SR

1.3.1. Mechanism of calcium accumulation

Calcium accumulation by SR vesicles, isolated from skeletal muscle is coupled stoichiometrically to ATP hydrolysis by a membrane-bound (Mg^{2+} , Ca^{2+}) - ATPase. Hydrolysis of ATP

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

The membrane of the SR exhibits two types of ATP hydrolysis namely Ca^{2+}-independent and Ca^{2+} -dependent hydrolysis. Both these ATPase activities require Mg^{2+} for full activation. However the Ca^{2+} -independent activity also known as the basal ATPase, does not require Ca^{2+} for activation. The Ca^{2+}-dependent or extra ATPase is absolutely dependent on Ca^{2+} concentrations between 0,01 and 1 μM (Hasselbach and Makinose, 1961 as quoted by Tada et al., 1978). Basal ATPase activity is abolished when the sarcoplasmic reticulum membrane is solubilized by detergents (Mc Farland and Inesi, 1970). Ca^{2+} -activated ATP hydrolysis also termed 'extra ATP splitting' by Hasselbach and Makinose (1961), is coupled to the uptake of Ca^{2+} by SR vesicles. When the extravesicular Ca^{2+} concentration is reduced to less than 0,01 μM due to calcium uptake by the vesicles, ATP hydrolysis reverts to a state which is slightly greater than 'basal' rate. If SR vesicles are made leaky by treatment with detergent (Mc Farland and Inesi, 1970) or EGTA at alkaline pH (Duggan and Martonosi, 1970), a high rate of Ca^{2+} dependent ATP hydrolisis is maintained for a considerable time. The Ca^{2+} -dependent ATPase activity is therefore closely related to calcium accumulation by SR vesicles.

The energy required for the active transport of Ca^{2+} by SR is derived from the hydrolysis of ATP (Martonosi, 1971) acetylphosphate (De Meis, 1969) or carbamyl phosphate (Puccel and Martonosi, 1971). The hydrolysis of ATP or acetylphosphate occurs with the formation and decomposition of a phosphoprotein intermediate EP. The hydrolysis of EP and the subsequent production of phosphate (Pi) occurs concommitantly with Ca^{2+} accumulation and occurs as long as accumulation is supported by energy yielding phosphate compounds. Ca^{2+} accumulation, coupled to extra ATP splitting, diminishes when the level of calcium is reduced to

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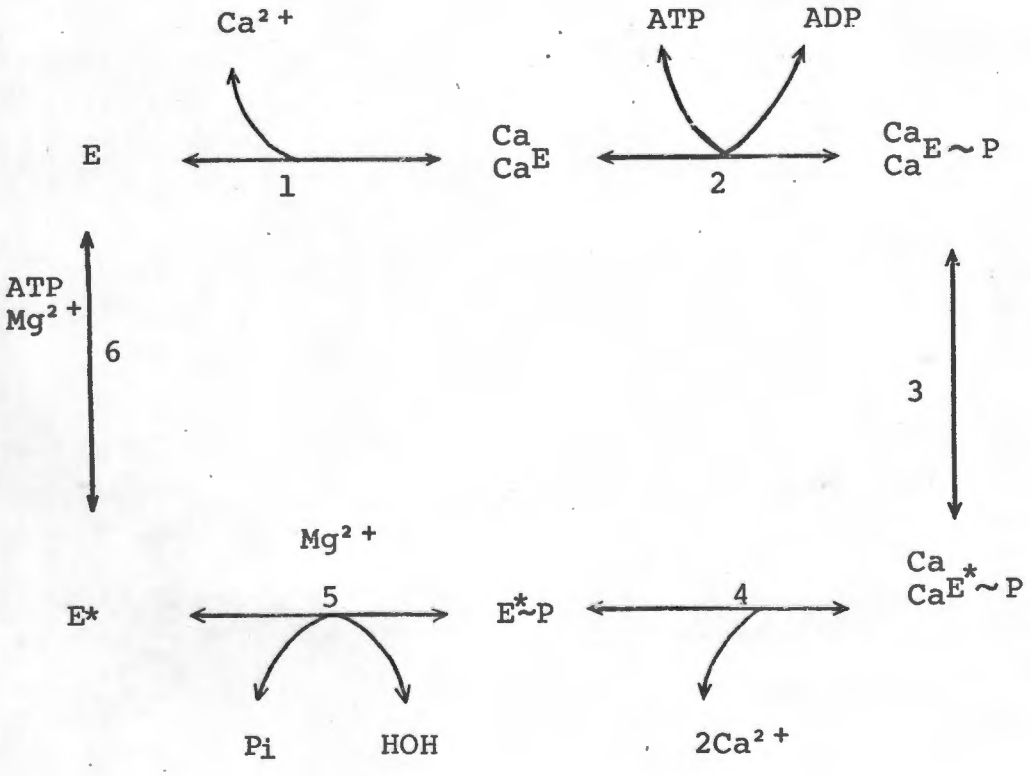
below 10 nM. For a number of energy yielding substrates, namely p-nitrophenyl phosphate, ATP and acetylphosphate, Ca^{2+} transport is coupled to calcium activated extra splitting with a 2:1 molar ratio. In agreement with the calcium transport ratio of 2, the dependence of calcium transport and ATP splitting on the concentration of ionised calcium is characterised by a Hill coefficient of 2 (Hasselbach, 1978). In 1976, Carvalho et al. proposed a reaction sequence of Ca^{2+} accumulation based on accumulated evidence. In this reaction sequence, the Ca^{2+} - dependent ATPase is represented in two different conformations, E and E*. In the E form, the site which translocates Ca^{2+} through the membrane faces outwards from the surface of the membrane and has a high affinity for Ca^{2+} with an apparent Kd $\simeq$ 1 - 3 µM at pH7,0. In the E* conformation, the Ca^{2+} binding site faces the vesicle lumen and has a low affinity for Ca^{2+} with an apparent Kd $\simeq$ 1 - 3 mM at pH 7,0.



Scheme of Ca^{2+} translocation in SR as proposed by Carvalho et al., 1976.

It has been assumed that formation of the phosphoprotein occurs on the outside of the vesicles (steps 1 - 2). This is followed by the translocation of Ca^{2+} to the membrane interior (step 3), release of Ca^{2+} from the enzyme (step 4), hydrolysis of the phosphoprotein (step 5) and interconversion of the enzyme from the E* to the E conformation with the high affinity Ca^{2+} binding site again facing the outside of the membrane (step 6). E* can be phosphorylated by Pi but not by ATP. The interconversion of E* to E (Step 6) is the slowest reaction of the sequence. ATP activates the interconversion of E* to E (Carvalho et al., 1976). The process of Ca^{2+} transport is fully reversible and ATP synthesis is coupled with the release of accumulated Ca^{2+} from the SR (Martonosi et al., 1974). The synthesis of ATP is initiated by the phosphorylation of E* by Pi (Step 5). Masuda and de Meis (1973) demonstrated the reversibility of step 5 by measuring the disappearance of phosphoenzyme when the Pi concentration of the medium decreased. Boyer et al., (1977) demonstrated the reversibility of step 5 by measuring the rate of the $Pi \rightleftharpoons HOH$ exchange which represents dynamic reversal of step 5. Boyer and collaborators also showed that the rate of interchange of phosphate between Pi of the medium and E*~ P under steady state conditions is rapid with a half-time for 50% substitution of the E*~ P phosphate in the range of 30 to 50 msec. They further showed that both the phosphorylation by Pi and the $Pi \rightleftharpoons HOH$ exchange occur in the absence of a transmembrane Ca^{2+} concentration gradient (Boyer et al., 1977).

Phosphorylation of the enzyme by Pi requires that the outer surface of the vesicle membrane be exposed to a medium where the Ca^{2+} concentration is very low. If the Ca^{2+} binding site of high affinity is saturated (step 1), E* will be converted slowly into E(step 6) and the Pi unreactive form $^{ca}_{ca}E$ will accumulate (deMeis and Tume, 1977). de Meis and Tume (1977) postulated that each of the individual reactions involved in the synthesis of ATP is reversible and can flow backwards or forwards without the utilization of energy derived from a transmembrane Ca^{2+} concentration gradient. The authors also conclude that the rate of either ATP synthesis or ATP hydrolysis in the presence of Mg^{2+}

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depends on the asymmetrical binding of calcium on the two sides of the membrane and on the relative concentrations of Pi, ADP and ATP in the medium. When calcium binds only to the site of low affinity, step 1 becomes irreversible and ADP plus Pi drive the reaction in the direction of ATP synthesis. Conversely when calcium binds only to the site of high affinity, step 4 becomes irreversible and ATP drives the reaction in the direction of ATP hydrolysis. When Ca^{2+} binds to both the high and the low affinity sites, the enzyme simultaneously catalyses the synthesis and hydrolysis of ATP which leads to ATP ⇌ Pi exchange. During this ATP ⇌ Pi exchange reaction the ratio between hydrolysis and synthesis of ATP depends on the relative concentrations of ATP, ADP and Pi in the medium (Carvalho et al., 1976). In sealed vesicles actively synthesising ATP, the large difference in Ca^{2+} concentrations across the membrane is there solely to meet the differences in affinities of the external and internal Ca^{2+} -binding sites.

Calcium dependent hydrolysis of ATP by SR vesicles requires the presence of both Ca^{2+} and Mg^{2+} for full activation . At Ca^{2+} concentrations above 0,01 µM in the presence of saturating concentrations of Mg^{2+} (equimolar to ATP), the rate of ATP hydrolysis increases with increasing Ca^{2+} concentration reaching maximal velocity at about 1 µMCa^{2+}. Calcium at concentrations above 100µM inhibits ATP hydrolysis (Tada et al., 1978). Magnesium has at least two important roles in ATP hydrolysis by sarcoplasmic reticulum. One is to accelerate the decomposition of the phosphorylated intermediate formed during calcium transport and the other is to form an equimolar complex with ATP and serve as the true substrate of the Ca^{2+} -dependent ATPase (Vianna, 1975). Vianna (1975) demonstrated that the Lineweaver -Burk plot of the Ca^{2+} -dependent ATPase activity in the presence of equimolar concentrations of Mg^{2+} and ATP was linear only when plotted against the reciprocal of the Mg ATP complex.

The membranes of the sarcoplasmic reticulum catalyse a phosphate exchange between ADP and ATP in the presence of Ca^{2+} and Mg^{2+} . Hasselbach and Makinose (1961) postulated that a high energy

phosphoprotein was formed as an intermediate of the reactions during active transport of Ca^{2+} on the basis that the rapid exchange of phosphate exhibited the same dependence on Ca^{2+} concentration as did ATP hydrolysis and calcium uptake by the same membranes. Phosphoprotein levels at the steady state depend on the concentrations of Ca^{2+} and ATP and parallel those of Ca^{2+} -dependent ATPase activity. The pH profile of the phosphoprotein is similar to that of the Ca^{2+} -dependent ATPase activity (Tada et al., 1978) suggesting that the phosphorylated protein is a true intermediate of the Ca^{2+} -dependent ATPase reaction. When calcium is translocated from the outside to the inside of the membrane, it has to be released from the enzyme into the vesicular lumen. For Ca^{2+} to be translocated into the vesiclar lumen a minimum of 3 steps are required. Ca^{2+} should be bound with high affinity on the external surface of the vesicle, followed by its translocation across the membrane and subsequent release on the inside of the vesicle. To facilitate this release the enzyme should undergo a change in its affinity for Ca^{2+} i.e. from the high affinity binding on the outside to low affinity binding on the inside. Such a cyclical behaviour of the calcium binding site has been reported. Using the technique of equilibrium dialysis Ikemoto (1974;1975) has shown that the solubilised (Ca^{2+},Mg^{2+}) ATPase binds Ca^{2+} with high (μ M) affinity. Phosphorylation of the enzyme is accompanied by a concomitant decrease in the enzyme's affinity for calcium. Following dephosphorylation the ATPase once again binds Ca^{2+} with high affinity.

High (mM) concentration of calcium inhibit ATPase activity by preventing the dephosphorylation of EP (Martonosi, 1968) . De Meis and coworkers have postulated that binding of Ca^{2+} to the low affinity (mM) site prevents this dephosphorylation (de Meis and Carvalho, 1974; de Meis and Sorensen, 1975); an alternative explanation for the inhibition of ATPase activity by high concentrations of Ca^{2+} is that Ca^{2+} inhibits the decomposition of EP by competing at the site where Mg^{2+} accelerates this decomposition (Yamada and Tonomura, 1972).

1.3.2./...

1.3.2. Mechanisms of calcium release from SR.

1.3.2.1. Activated calcium release

Makinose et al. (1971) observed that a fast calcium release was induced by the addition of sodium or potassium phosphate to vesicles loaded with calcium oxalate, if ATP was the energy donor. Barlogie and collaborators (1971) also observed that phosphate was ineffective as a calcium - releasing agent after SR vesicles were loaded with calcium if acetyl phosphate was the energy donor instead of ATP. Also, the addition of ADP to acetyl phosphate loaded vesicles was found the restore the phosphate response. The phosphate - induced efflux of Ca^{2+} from actively loaded vesicles therefore shows an ADP dependence suggesting reversal of ATP -dependant calcium uptake. In agreement with this concept, ADP - and phosphate-dependent calcium release require Mg^{2+} as does the ATP - driven calcium uptake.

1.3.2.2. Calcium release-coupled to ATP synthesis

Makinose and Hasselbach (1971) have demonstrated calcium efflux-coupled to net synthesis of ATP thus confirming the concept of reversal of the pump. ATP synthesis can be demonstrated in SR vesicles passively loaded in solutions containing 10 - 100mM $CaCl_2$ (Makinose, 1972) or actively loaded with ITP or acetyl phosphate in the presence of the calcium precipitating anions phosphate or oxalate (Barlogie et al., 1971). The calcium load resulting from passive loading does not exceed 50 - 100 nmol/mg. This amount is released in approximately 1 min, while the internal concentration of free Ca^{2+} steadily declines. In contrast, a constant internal free calcium concentration is maintained after active calcium loading because in the presence of an appropriate and constant oxalate or phosphate concentration in the medium the internal calcium concentration is constant as long as calcium precipitates are present inside the vesicles. In the presence of precipating anions, a calcium load of 800 - 1000 nmol Ca^{2+}/mg can be achieved (Hasselbach, W. 1978). Under these conditions the release rate is strongly dependent on the concentration of phosphate or oxalate in the

medium/...

medium, with phosphate giving considerably higher efflux rates than oxalate (Hasselbach, 1978). Calcium efflux-coupled synthesis of ATP occurs under conditions where calcium inside the vesicles (Cai) is high and calcium outside the vesicles (Ca_o) is low. Calcium release and concommitant ATP synthesis therefore occurs in an assay medium containing phosphate (Pi), ADP, Mg^{2+} and EGTA. The latter is included in order to reduce the level of ionised calcium below 0.1 µM. Release can be initiated by the addition of any of the reagents to assay mediums containing the other three obligatory reactants. This process, in which osmotic energy derived from the downhill flux of Ca^{2+} is used to drive ATP synthesis (Barlogie, et al., 1971), is inhibited by high Ca_o and by high levels of ATP in the surrounding medium (Katz et al., 1977). Calcium release and ATP synthesis cease when the external calcium level is raised from 0,1 to 1µM; the same concentration range in which ATP-dependent transport is activated. This inhibition is presumably caused by occupation of external calcium-binding sites which prevents calcium efflux and ATP synthesis (Hasselbach , 1978).

Calcium efflux and ATP synthesis are strictly coupled. For every two Ca^{2+} ions that leave the vesicle, one phosphate group is transferred from ADP to ATP (Makinose and Hasselbach, 1971). Neither azide nor 2,4-dinitrophenol and m-chlorocarbonyl-cyanide phenylhydrazone (CCCP) interfere with calcium efflux-coupled synthesis. The ionophores, gramicidin and valinomycin, which affect proton gradient-driven ATP synthesis or ion translocation in mitochondria are ineffective in interfering with efflux-coupled ATP synthesis. Apart from calcium ionophores, the only agent which effectively uncouples calcium gradient driven ATP synthesis is arsenate (Hasselbach et al., 1972).

1.3.2.3. Passive efflux from SR vesicles.

Liposomes as well as sarcoplasmic reticulum vesicles become highly permeable for calcium when lipophilic calcium ionophores like X537A and A23187 are incorporated into the membranes (Scarpa and Inesi, 1972). Both X537A and A23187 act as divalent cation ionophores and their addition to previously-loaded SR vesicles

leads/...

leads to immediate release of Ca^{2+} (Entman et al., 1972), while neither ATPase inhibition nor structural damage to the vesicles is apparent (Scarpa and Inesi, 1972).

1.4. The effects of detergents on biological membranes

Deoxycholate is more effective than non-ionic detergents in removing protein bound lipid and has formed the basis for the purification of the (Ca^{2+},Mg^{2+}) - ATPase (section 1.5). In reconstitution experiments the only criterion a detergent must conform to is that its effect be reversible. The protein need not have its native conformation in the presence of the detergent but must be able to reacquire its conformation upon its removal. (Le Maire et al., 1976).

Helenius and Simons (1975) have summarised the events that occur upon the addition of increasing concentrations of detergents to biological membranes. Briefly, when the molar ratio of detergent to lipid is low, the detergent is incorporated into the membrane without disrupting it. Further addition of detergent may disrupt the membrane and lead progressively to soluble membrane fragments containing many protein molecules and/or protein-lipid-detergent complexes containing a single protein and/or lipid-free detergent-protein complexes (Helenius and Simons, 1975). These successive stages of solubilization may occur with some proteins without evident structural changes, for example microsomal cytochrome b5 appears to retain its active site and other major structural features in lipid-free complexes with a number of mild detergents (Robinson and Tanford, 1975; Visser et al., 1975).

In contrast, the sarcoplasmic reticular (Ca^{2+}, Mg^{2+}) ATPase is more sensitive to its immediate environment. Solubilization of the ATPase by sodium deoxycholate is accompanied by loss of enzymatic activity (Martonosi, 1968) and if solubilization is accompanied by delipidation, the inactivation becomes irreversible (Hardwicke and Green 1974). Non-ionic detergents, e.g.

Triton/...

Triton X100, have been used to solubilize and purify the (Ca^{2+},Mg^{2+}) - ATPase (Mc Farland and Inesi, 1970; Ikemoto et al., 1971) The danger of irreversible inactivation appears to be much less with Triton X100 than with Na-deoxycholate but since these detergents are used under varying conditions it is difficult to compare their effects (Warren et al., 1974 a,b ; Petersen et al., 1978). Removal of detergent from solubilised protein, while lipid is present, results in the formation of vesicular preparations which exhibit both ATPase and calcium transport activity (Martonosi, 1968; Meissner et al., 1973; Warren et al., 1974 a,b).

The individual methods of reconstitution, the effects of detergent used as well as the characteristics of the reconstituted vesicle will be discussed in more detail below (Section 1.5.).

1.5. Reconstitution

Sarcoplasmic reticulum membranes are composed of various kinds of lipids and proteins. It is interesting therefore to define which components are essential for calcium transport by the intact membrane. Studies of the functions and properties of individual proteins and lipids led to the development of model membrane systems consisting of lipid bilayers into which single proteins of interest could be incorporated.

The value of reconstituted membranes of ion-translocating proteins lies beyond demonstrating that a given protein is indeed an ion-translocator. Proteins are integral components of biological membranes and are intimately associated with the membrane lipids. Lipid-protein interactions are critical not only from a structural aspect but also from a functional one since the activity of the protein is dependent on physio-chemical characteristics of its lipid environment (Warren et al., 1974; Hesketh et al., 1976).

Reconstitution/...

Reconstitution experiments have followed two general approaches; (1) vesicles limited by a single or multiple lipid bilayer also referred to as liposomes (Racker et al., 1975). or (2) Black lipid membranes as described by Shamoo et al., 1977) to study ionophoric activities of individual peptides isolated from the 102K dalton (Ca^{2+}, Mg^{+}) -ATPase by tryptic digestion. Liposomes are formed by the simultaneous assembly of protein and lipid. Their formation requires removal, in the presence of excess phospholipid of the detergent in which the protein has been solubilised. The phospholipid can either be exogenous in origin in which case a purified ATPase is used (Knowles and Racker, 1975) or endogenous in origin (Repke et al., 1976). Exogenously added lipid has been used in systems where the effect of specific lipids on ATPase and calcium transport activities has been investigated (Knowles and Racker, 1975). Detergents can be removed by slow dialysis (Kagawa and Racker, 1971), dilution (Racker et al., 1975) or by sephadex G-25 chromatography (Martonosi, 1968). The individual methods of reconstitution will be discussed below.

1.5.1. Review of the methods of reconstitution

Martonosi et al., (1968) presented evidence that selective degradation by phospholipase C of lecithin bound to SR membranes resulted in inhibition of ATPase activity and calcium transport function. Martonosi and coworkers were able to reconstitute both ATPase and calcium transport activity in lecithin depleted sarcoplasmic reticulum membranes by the addition of exogenous lipids. They found that the structural requirements for the activation of calcium transport and ATPase activity were considerably different. ATPase activity of phospholipase C-treated SR vesicles was reconstituted with synthetic lecithin, lysolecithin , phosphatidic acid and nonionic detergents, such as Triton X100, Tween 20 and Brij -35. Reactivation of calcium transport in phospholipase C-treated sarcoplasmic reticulum required lecithin, lysolecithin or phosphatidic acid. Detergents

were/...

were found to be ineffective in the reactivation of phospholipase C-treated sarcoplasmic reticulum. The range of phospholipid concentration in which activation occurs is sharply defined. High phospholipid concentrations caused inhibition of ATPase activity and calcium transport, accompanied by solubilisation of SR vesicles, especially in the case of high lysolecithin concentrations (Martonosi et al., 1968). In an accompanying paper Martonosi (1968) extended these findings to liposomes reconstituted from solubilised sarcoplasmic reticulum membranes. The membranes were solubilized with deoxycholate and spontaneously formed vesicles upon removal of the deoxycholate by Sepahdex G25 chromotography. Martonosi observed essentially the same results in the reconstituted vesicles as found in the native vesicles, namely that phospholipase C-treatment caused inhibition of ATPase activity and calcium transport which could be reversed by the addition of lecithin and lysolecithin. From these results Martonosi concluded that the deoxycholate-reconstituted vesicles exhibited the same structural and physico-chemical properties as their native counterparts.

The purification of the major protein component of sarcoplasmic reticulum namely the (Ca^{2+} , Mg^{2+})-ATPase was first achieved by Mac Lennan (1970) who treated the sarcoplasmic reticulum membrane with 0.1 mg Deoxycholate/mg protein in the presence of 1M KCl. The combined effect of the low deoxycholate concentration and the high salt concentration served to eliminate most of the extrinsic and contaminating proteins. The ATPase protein remaining in the membrane was subsequently dissolved with higher concentrations of deoxycholate (0.5mg DOC/mg protein) and fractionated with ammonium acetate. Mac Lennan found that the specific activity of the ATPase increased with protein purification. The enzyme, which was soluble by virtue of bound detergent, became insoluble when the deoxycholate was removed. Mac Lennan showed that both the insoluble and soluble forms of the enzyme were stable with respect to ATPase activity under appropriate storage conditions. Furthermore, the purified ATPase was found to exhibit all the features of the enzyme in the intact membrane. The amount of phospholipid bound to the enzyme was comparable

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in amount to that found in intact membranes. The purified enzyme catalysed an ATP ⇌ ADP exchange and was phosphorylated by $[\gamma - P^{32}]$ -ATP. In addition, the rate of the ATP ⇌ ADP exchange and the level of phosphorylation were both increased by a factor commensurate with the increase in ATPase activity. In agreement with the results of Martonosi (1968) and Martonosi et al., (1968), Mac Lennan (1970) found that the ATPase activity of the purified ATPase was inhibited by phospholipase C digestion but that the inhibition could be reversed by addition of phospholipid, thus implicating phospholipid in playing a major role in the functioning of the purified ATPase.

Similar attempts at purifying the ATPase protein were made by Ikemoto et al. (1971a) who solubilised SR membranes with Triton X100. The ATPase was subsequently purified by sephadex chromatography. Deoxycholate treatment was also used by Meissner and Fleischer (1971) and Warren et al. (1974 a). The method of Warren et al. (1974a) was time-and effort- saving and depended on the treatment of SR membranes with 10% DOC and subsequent purification of the (Ca^{2+}, Mg^{2+}) ATPase by centrifugation through a sucrose-gradient. These authors reported a value of 0.3% DOC bound to the purified enzyme.

Studies on sarcoplasmic reticulum up to this stage (i.e. 1971) were performed on intact membranes or membranes solubilised with detergent and subsequent reconstitution with endogenous lipids. Since then the isolated and purified (Ca^{2+}, Mg^{2+})-ATPase has been incorporated into single bilayer phospholipid vesicles. These reconstituted Ca ATPase liposomes carry out both Ca^{2+} - and Mg^{2+} - dependant ATP hydrolysis as well as ATP-dependent transport of Ca^{2+} ions thus demonstrating that the Ca ATPase is an energy dependent Ca^{2+} translocator (Korenbrot, 1977).

In 1972, Racker first reported the formation of Ca-ATPase liposomes using a cholate dialysis procedure. The Ca-ATPase was previously purified and added to a sonicated suspension of soybean phospholipid in cholate. The cholate was subsequently removed by slow dialysis and vesicles, capable of calcium

transport/...

transport, ATP hydrolysis and Pi $\rightleftharpoons$ ATP exchange, were formed. The efficiency of Ca transport, measured as the mole ratio of Ca transported to ATP hydrolysed in these liposomes has been found to be generally less than those in native sarcoplasmic reticulum membranes. In 1973 Racker described a method for the rapid reconstitution of biologically active phospholipid vesicles without the use of detergents. The method involved the suspension of dried phospholipids in salt solution containing the membrane proteins to be studied. The whole mixture was then subjected to sonic oscillation for varying lenghts of time. Using this method Racker (1973) was able to reconstitute Pi $\rightleftharpoons$ ATP exchange in mitochondria, cytochrome oxidase vesicles exhibiting respiratory control and Ca-ATPase liposomes exhibiting ATPase activity and calcium transport. The method could thus be applied to a number of systems.

Meissner and Fleischer (1973) introduced a new approach in the reconstitution of Ca ATPase liposomes. Sarcoplasmic reticulum membranes were dissolved with deoxycholate. The solubilized protein contained endogenous lipid of the same concentration and composition as intact sarcoplasmic reticulum. The soluble fraction, i.e. the supernatant resulting from the removal of insoluble protein at 200 000 xg, was reconstituted into particulate liposomes by removal of the deoxycholate by slow dialysis at room temperature. The resulting vesicles had a density and structure similar to that of native vesicles and showed the same rate of formation of phosphorylated intermediate. However the ATP hydrolytic activity became uncoupled from Ca^{2+} transport. The Ca^{2+} -dependent ATPase activity was four-fold that of native membranes whereas Ca^{2+} transport in the presence of oxalate was only half that of native sarcoplasmic reticulum. Repke et al. (1976) used a similar procedure to that of Meissner and Fleischer (1973). They used lower salt and DOC concentrations than those used by Meissner and Fleischer and retained more than 85% of the 55K and 18 % of the 45K dalton Ca^{2+} binding proteins. Up to 90% of the calcium storage capacity measured in the absence of calcium precipitating anions was retained. In contrast, Meissner and Fleischer (1973) using higher salt and DOC concentrations recovered only

25%/..

25% of the calcium storage capacity in reconstituted vesicles. Loss of the Ca^{2+} storage capacity coincided with loss of most of the larger (i.e. 55 000 fraction) of the calcium binding proteins.

Recovery of a tightly coupled calcium pump in reconstituted sarcoplasmic reticulum depends on conditions during both solubilization and reconstitution. DOC concentrations greater than 5mM solubilize virtually all of the protein but yield less active vesicles. The absolute concentration of DOC as well as the ratio of DOC to protein influences the properties of reconstituted vesicles. Inclusion of KCl during DOC treatment increases the amount of protein solubilized but like the higher detergent concentrations, high KCl concentrations reduce calcium transport of reconsituted vesicles (Repke et al., 1976).

A cholate-dilution technique was developed by Racker et al. (1975) for the reconstitution of Ca ATPase liposomes. This method involves the sonication of phospholipids either in the presence or absence of cholate and mixing of the sonicated suspension with the membrane proteins at a final concentration of 0.7% cholate. After an optimal time of incubation (usually 20 min at 4°c) the sample is diluted 25 fold into an assay mixture. The major advantage of the cholate - dilution procedure as described by Racker et al. (1975) is rapidity and reproducibility. In this method protein is added to sonicated phospholipid suspension instead of the protein-phospholipid mixtures being sonicated together . The latter method (Racker, 1973) is prone to variability caused by difficulties in controlling sonication (Racker, 1975). Whereas isolated sarcoplasmic reticulum (Hasselbach and Makinose, 1961) or vesicles re-formed from their original components (Meissner and Fleischer, 1974; Repke et al., 1976) accumulate large quantities of calcium in the presence of oxalate or phosphate, vesicles reconstituted with purified Ca^{2+}-ATPase and

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added phospholipid require internally trapped phosphate or oxalate (Racker, 1972; Racker and Eytan, 1973). Such vesicles reconstituted with exogenous lipid as described by Knowles and Racker (1975) were found to be impermeable to ^{14}C oxalate, ^{3}H- or ^{32}P-labelled ATP or ^{32}Pi and only transported Ca^{2+} when reconstituted in the presence of the calcium sequestering agents oxalate or inorganic phosphate. When these vesicles were assayed for ATP-independent transport activity, high values of Ca^{2+} uptake, proportional to the concentration of the potassium buffers used (up to 0.4 M KPi), were found. Since an increase in the potassium concentration of the assay medium markedly decreased the ATP-independent Ca^{2+} uptake, it seemed likely (Knowles and Racker, 1975) that Ca^{2+} uptake in the absence of ATP was a manifestation of a potassium gradient created by differences in intra- and extravesicular ion concentrations.

Warren et al. (1974 a,b) found that endogenous lipid can be decreased from the Ca-ATPase in the original 80:1 phospholipid: protein mole ratio in native SR to a 30:1 ratio without loss of ATPase activity. Below a 30:1 ratio , enzymatic activity decreases linearly with decrease in the value of this ratio until activity is irreversibly lost at a ratio 15:1 (phospholipid: protein). Warren (1974b) suggested that the functional stability of the ATPase only at mole ratios higher than 30:1 is the result of the structural need of the protein to be surrounded in the membrane by a bilayer of phospholipid one molecule in thickness. Warren and collaborators (1974a,b) developed a method for phospholipid exchange which allows complete control of the lipid environment of the protein without depletion of the lipid around the protein. The method depends on the equilibration of endogenous lipid with a pool of excess added lipid in the presence of cholate and subsequent recovery of the ATPase/lipid complex by centrifugation into a sucrose gradient. Using this method, over 99% of the endogenous lipid can be substituted by known lipid. Warren et al. found that ATP hydrolysis by the ATPase is regulated by specific interactions between protein and phospholipid. These interactions were found to be specified by the fatty acid moiety of the phospholipid and not by the polar head groups. Thus dioleoyl lecithin and dioleoyl phosphatidylethanolamine

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have about the same effect on the ATPase. Dioleoyl phosphatidyl choline (di 18:1) was more effective in supporting ATPase activity than distearoyl phosphatidylcholine (di 18:0) and both were more effective than dimiristoyl lecithin (di 14:0). The nature of the fatty acid moiety with respect to chain length and degree of saturation thus plays a role in the support of ATPase activity. Korenbrot (1977) states that the fatty acid must be unsaturated, be at least 14 carbons long and have a double bond more or less in the middle of the chain to effectively restore ATPase activity.

The effect of phospholipids on Ca^{2+} -transport activity is less well defined. Warren et al. (1974 a) reported that Ca-ATPase liposomes formed from dioleoyl phosphatidylcholine (DOPC) transport calcium in the presence of ATP but Racker et al.(1975) and Knowles and Racker (1975a) did not obtain similar results. Using a preparation of Ca ATPase with endogenous lipid, Knowles et al. (1975b) found that Ca ATPase liposomes transport Ca^{2+} only when formed with soybean phosphatidylethanolamine (PE) and that acetylation of PE led to inhibition of Ca^{2+} transport. Transport could then be reactivated by adding stearylamine or oleylamine and less effectively by the addition of myristoylamine or laurylamine. Knowles et al. (1975) also found that phosphatidylethanolamine was more effective than phosphatidylcholine in restoring Ca^{2+} transport activity, even though native sarcoplasmic reticulum contains about 65% phosphatidylcholine and 14% phosphatidylethanolamine (Knowles et al., 1975). Surprisingly vesicles with Ca^{2+} transport activity could be reconstituted with PE alone whereas vesicles reconstituted from PC alone possessed no Ca^{2+} transport activity. This is in contrast with earlier results (Racker and Eytan, 1973) where both PC and PE were required for Ca^{2+} transport activity when vesicles were formed by the sonication procedure.

Knowles et al. (1976) have used delipidated Ca^{2+} ATPase and purified phospholipids to reconstitute vesicles capable of translocating Ca^{2+}. In agreement with their earlier results

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(Knowles et al., 1975) they found that phosphatidylethanolamine alone was sufficient in restoring Ca^{2+} transport activity whereas phosphatidylcholine alone was not. Phosphatidylcholine however was sufficient in reactivating adenosine triphosphatase activity in the delipidated Ca^{2+} ATPase. In order to test the effect of phospholipid headgroup structure on the activity of the ATP-dependent calcium pump, Bennet et al. (1978) replaced the lipid associated with purified Ca-ATPase with defined synthetic dioleoyl phospholipids. These assays were performed at 37°C, well above the liquid / crystalline to crystalline phase transition for these lipids. Bennet and coworkers found that the zwitterionic phospholipids, dioleoyl phosphatidyl choline and dioleoyl phosphatidylethanolamine (DOPE), supported the highest ATPase activity while dioleoyl phosphatidic acid with two negative charges supported on activity twenty times lower. Dioleoyl phospholipids with single net negative charges supported an intermediate ATPase activity, not affected by the precise chemical structure of the phospholipid headgroup. The charge structure of the phospholipid headgroup was thus interpreted as being the dominant factor in determining the phospholipid headgroup specificity of the Ca^{2+} pump. The authors also determined whether the lipid specificity for Ca^{2+} translocation was the same as that for the Ca^{2+} -dependent ATPase activity. Purified Ca-ATPase was reconstituted into vesicles comprising different phospholipids using an adaptation of the cholate dialysis procedure of Racker (1972). They found that while all the test lipids supported significant ATPase activity, three of them namely dioleoyl phosphatidylethanolamine, N-acetyl dioleoyl phosphatidic acid and O-Methyl dioleoyl phosphatidic acid were unable to support calcium accumulation. Furthermore the inability to accumulate calcium could not be correlated with the chemical structure or charge on the phospholipid headgroup. For example, dioleoyl phosphatidylethanolamine having the same headgroup charge as dioleoyl phosphatidylcholine, did not support calcium accumulation at all while dioleoylphosphatidyl choline supported calcium accumulation of the order of 0,84 µmol/min.mg at 25°C. Electron microscopy of the reconstituted vesicles revealed that those phospholipids, unable

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to support Ca^{2+} accumulation, did not form closed vesicles but when mixed with dioleoyl phosphatidylcholine these lipids were able to form sealed vesicles. These results of Bennet et al. (1978) are in contrast with those of Racker and co-workers who observed calcium uptake in reconstituted vesicles, comprising phosphatidylethanolamine from soya lipids, whereas phosphatidyl choline was ineffective. The results of Bennet et al. do however agree with those of Racker and coworkers (1975) who found that mixtures of phosphatidylethanolamine and phosphatidylcholine were more effective than phosphatidyl-ethanolamine alone and that calcium uptake was not observed in vesicles reconstitued with N-acetyl phosphatidylethanola-mine (Knowles et al., 1975).

The action of lysolecithin, Triton X100 and cholate on the calcium transport ATPase of lobster SR was investigated by Petersen et al. (1978) who found that lysolecithin was effec-tive in releasing the ATPase for subsequent purification but did not promote the exchange of membrane lipid classes. This was in contrast to the action of Triton X100 and cholate which promoted the exchange of membrane phospholipids. High concen-trations of Triton and cholate were found to inhibit the ATPase activity but the effect could be reversed by the addition of phospholipids or fatty acids directly to the mixture. Petersen et al. showed that under their conditions reactivation de-pended on the presence of lipid acyl chains rather than speci-fic headgroups. Particulate liposomes able to transport Ca^{2+} were formed by passing Triton X100 solubilized protein through a hydrophobic bead column.

It would appear from the above review of the methods of recon-stitution that the conditions for the reconstitution of ATPase activity and calcium transport activity are essentially dif-ferent with specific lipid-protein interactions determining the correct conformation of the ATPase for both ATP dependent calcium transport activity and Ca^{2+} -dependent ATPase activity. The succeeding section (1.6) will review current concepts of lipid-protein interactions and will illustrate the role of lipid -protein interactions in the functioning of the (Ca^{2+} ,Mg^{2+})ATPase.

1.6/....

1.6. Lipid-protein interactions in the SR membrane

The present view of biomembrane structure is that of a lipid bilayer forming the basic matrix into which various proteins are fitted. In most cases the lipid matrix is in a fluid condition with all the lipids essentially above their Tc transition temperature and able to diffuse along the bilayer length (Chapman et al., 1979). The presence of intrinsic proteins in biomembranes raises questions concerning the degree and type of perturbations which such proteins exercise on their surrounding lipid environment and vice versa (Fernandez et al., 1979).

The Ca-ATPase molecule is amphiphilic and simultaneously interacts with the hydrophobic core of the lipid bilayer and the aqueous environment of the membrane. The amphiphilic characteristic of the enzyme is fundamental to its function, the enzyme being active only when it finds itself in its proper amphiphilic environment. In the sarcoplasmic reticulum membrane, cholesterol, phospholipids and fatty acids define the amphiphilic environment of the Ca-ATPase. They are required to maintain the integrity of the membrane since membranes made leaky to Ca^{2+} by mild treatment with diethylether (Inesi et al., 1967) or with phopholipase A (Fiehn and Hasselbach, 1970) show Ca-dependent ATPase activity without net Ca^{2+} transport. In other words sarcoplasmic reticular membranes can only exhibit Ca^{2+} transport if they are cabable of maintaining a permeability barrier to Ca^{2+}. Depletion of phospholipid of SR membranes by treatment with organic solvents (Martonosi et al., 1968) or detergents such as Triton X100 (Walter and Hasselbach, 1973) and cholate under certain conditions (Meissner et al., 1973) leads to irreversible inactivation of the Ca-ATPase. On the other hand controlled depletion of phospholipids produces complete but reversible loss of ATPase activity (Feihn and Hasselbach, 1970; Hasselbach et al., 1975; Martonosi et al., 1968). In intact sarcoplasmic reticulum, phospholipids comprise a large part of the total lipid (about 80%), the remainder being neutral lipid mainly cholesterol). Although the bilayer forms the basic

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framework of the membrane, phospholipids tightly bound to the ATPase protein may also exist (Tada et al., 1978). Although this concept has recently been questioned (Chapman et al., 1979).

The existence of a restricted lipid annulus in the neighbourhood of an intrinsic protein was first described by Jost et al. (1973) for beef heart mitochondrial cytochrome oxidase. Complexes of the membrane with varying amounts of mitochondrial lipid were probed by electron spin resonance spectroscopy and the resulting spectra were shown to be consistent with the co-existence of both a rigid and 'bulk' lipid environment. Jost and collaborators interpreted their findings to indicate that a single layer of protein bound phospholipids is essential in maintaining the activity of the cytochrome oxidase as isolated from beef heart mitochondria. They further suggested that an annular layer of phospholipids in direct contact with the hydrophobic region of the protein provided a boundary between the 'bulk' lipid of the membrane and the protein itself, thus helping to maintain the protein in its proper conformation. The existence of a boundary lipid in the membrane of sarcoplasmic reticulum was first suggested by Warren and coworkers (1974 a,b) who found that approximately 30 mol of lipid were tightly bound per mol of ATPase forming a annulus surrounding the protein. When the purified ATPase having more than 30 mol lipid per mol was delipidated by increasing amounts of deoxycholate, full ATPase activity was maintained only above a molar ratio of 30:1. At lower ratios there was an irreversible loss of activity reaching negligible levels at about 15 lipid molecules for each mol of ATPase (Warren et al., 1974b). Cholesterol normally is excluded from the lipid annulus of the ATPase in the intact membrane, and the presence of cholesterol in the extra-annular environment does not affect Ca^{2+} -dependent ATPase activity as shown by Warren et al. (1975). Warren and coworkers (1975) further found that the presence of a high concentration of deoxycholate allowed cholesterol to replace the annular lipid causing complete but reversible inactivation of ATPase activity. Complete loss of activity was observed

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at 15 mol of cholesterol replaced per mol of ATPase (Warren et al., 1975). These findings suggest that about 30 lipid molecules interact directly with the hydrophobic part of each ATPase molecule to support full enzymic activity. Warren and coworkers (1975) interpret the reversible inactivation of ATPase activity by cholesterol as a function of the inability of cholesterol to meet the requirements of the protein for specific chemical structures in the annulus: for example β-hydroxybutyrate dehydrogenase specifically requires lecithin to bind the NAD^+ coenzyme (Houslay et al., 1975). Another interpretation forwarded by Warren et al. (1975) is the possibility that the very rigid interaction which occurs between cholesterol and the hydrophobic surface of the protein could also account for the inhibition of ATPase activity. This interpretation supports earlier work by Warren and coworkers (1974b) who showed that the rigid interaction which occurs between ATPase complexes and saturated lecithins at or below their thermal phase transitions is sufficient to completely inhibit the ATPase. The inhibition by both cholesterol and saturated lecithins can be partially relieved by perturbing agents, such as short chain alkyl alcohols which fluidise the lipid environemt of the protein (Warren et al., 1975).

Inesi and coworkers (1973) were the first to relate the temperature dependence of ATPase activity and calcium transport to the fluidity of the membrane bilayer lipid. They showed that the transition temperature of the temperature-activity profiles of calcium transport and ATPase activity of the sarcoplasmic reticulum was in good agreement with that of lipid and protein spin labels. They interpreted the observed temperature profile as representing a dual function of the phase transition of the bilayer phospholipids and the temperature dependence of the ATPase activity. This is in contrast to later findings by Hesketh et al. (1976) who found that ATPase activity was determined largely by the phase transition of the protein - bound annular lipid which was different from the bulk phospholipids in the fluid bilayer.

Hesketh/...

Hesketh and coworkers (1976) studied the temperature profile of the ATPase activity and lipid spin label in an ATPase preparation in which the endogenous lipid was replaced to varying degrees by dipalmityl lecithin (DPL). Pure complexes of DPL with the ATPase retained significant ATPase activity down to about 30°C, well below the transition temperature of pure DPL at 41°C. Above the minimal lipid requirements the complexes showed very similar dependence of the ATPase activity above 30°C. Spin - label studies demonstrated no DPL phase transition in the range of temperature between 30 and 48°C in complexes containing less than 30 DPL/ATPase, above which ratio a phase transition occurred at about 41°C . Hesketh et al. interpreted breaks in the Arrhenius plots of the ATPase activity occuring at 27 - 32°C and 38° C to indicate some cooperative structural properties of the interaction of the annular DPL molecules with the ATPase. These findings led them (Hesketh et al., 1976) to suggest that the annular phospholipids, by tight binding to the enzyme, exhibit a conformational property much altered from their inherent nature and that such an interaction of the ATPase protein with the annular lipids, but not with bilayer lipids, has a predominant effect in determining the form of the temperature - activity profiles (Hesketh et al., 1976).

Hidalgo et al. (1976) studied ATPase activity and mobility of spin-labelled stearic acids incorporated into ATPase enzymes containing either dioleyllecithin or dipalmitoyllecithin. They found ATPase activity and Ca^{2+} transport activity to be related to the fluidity of the membrane lipid reflected in the mobility of the spin labels; decreased fluidity of the lipid resulted in decreased ATPase activity. Studies on the formation and decomposition of phosphoenzyme showed that phospholipids affect primarily the dephosphorylation reaction. Replacement of endogenous phospholipids with dipalmitoyllecithin, resulted in strong inhibition of the rate of ATP hydrolysis at temperatures at which the mobility of the dipalmitoyllecithin molecules were severely restricted while the rate of phosphorylation was unaffected (Hidalgo et al., 1976). This is in agreement with earlier studies of Martonosi et al. (1968; 1971) and Meissner

and/...

and Fleischer (1972) who examined the effects of delipidation by phospholipases A and C and found that the formation of the phosphoprotein intermediate was not affected by delipidation, whereas ATP hydrolysis and calcium transport were markedly inhibited by delipidation. These results suggested that the decompostion of the phosphorylated intermediate of ATPase required the presence of phospholipids. In contrast, Feihn and Hasselbach (1970) reported decreased phosphoenzyme formation phospholipase A treated membranes. The observation of Martonosi et al. (1968) and Meissner and Fleischer (1972) were supported by those of Nakamura . M. and Ohnishi (1975) who reported that delipidation by phospholipase A caused commensurate losses of both ATPase activity and phospholipid content with no effect on phosphoenzyme levels.

Hardwicke and Green (1974) and Knowles et al. (1976) showed that complete delipidation of the purified ATPase resulted in complete loss of ATPase activity and phosphoenzyme formation. However, up to 50% of these activities were restored when the delipidated enzyme was recombined with phospholipids from various sources (Knowles et al., 1976).

Nakamura.H. and coworkers (1976) and Hidalgo et al. (1976) demonstrated that in an ATPase preparation whose phospholipids were substituted by synthetic phospholipids the decomposition of the phosphoprotein intermediate was influenced significantly by the nature and fluid state of the protein bound annular lipids whereas the formation of the phosphoprotein was not affected by these factors.

The results of Nakamura M. and Ohnishi (1975) suggest that the loss of ATPase activity was related to the loss of the fluid bilayer lipid. On the other hand Hesketh et al. (1976) interpret these results as modulation of ATPase activity by the annular lipid with bulk lipid playing a lesser role. Some difficulty exists in correlating the results of the two groups since Hesketh et al. (1976) failed to determine whether the decreased ATPase activity was due to a decrease in the formation or decomposition of the phosphorylated intermediate.

In reviewing the methods of reconstitution and protein-lipid interactions a distinct feature of the (Ca^{2+},Mg^{2+}) ATPase becomes apparent. The conditions for reactivating Ca^{2+} -dependent ATPase activity and ATP-dependent calcium transport activity are essentially different. ATPase activity appears to be dependent on the micro-environment of the protein. This has led to the formulation of two schools of thought, one favouring an annulus concept where the annular phospholipid determines the micro-environment and hence the ATPase activity (Warren et al., 1975; Hesketh et al., 1976), and another implicating the bulk phase phospholipids in regulating ATPase activity (Hidalgo et al.,1976; Inesi et al.,1973; Nakamura and Ohnisi, 1975).

In contrast the role of annular and/or bulk phospholipids in calcium transport is less defined. In addition conflicting evidence has been presented concerning the nature of the phospholipids able to support transport activity (Knowles et al., 1976; Bennet et al.,1978). A prerequisite of calcium transport activity is the ability of the SR vesicle to maintain a permeability barrier to Ca^{2+}. In contrast to ATPase activity, calcium transport activity depends on the integrity of the membrane. It is therefore probable that most of the phospholipids able to transport ATPase activity but not calcium transport activity do so by virtue of their inability to form closed vesicles.

2.0. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Isolation and Purification of Sarcoplasmic Reticulum

The method employed is a slight modification of the method described by Eletr and Inesi (1972). Sarcoplasmic reticulum was isolated from white skeletal muscle excised from the hind legs of a New Zealand white crossed with a commercial hybrid strain male rabbit. Immediately after excision the tissue was washed and cooled in 0.1mM EDTA, pH 7.0. 150g of trimmed muscle was homogenised in 450 ml of Medium I: 10mM Histidine, 10% sucrose, 0.1mM EDTA, pH 7.0 15 s every 5 min for 1h. During this procedure the pH was adjusted with a few drops of 5% ($^{w}/_{v}$) NaOH when necessary. The homogenate was centrifuged at 15000xg for 20 min, the supernatant collected and filtered through several layers of glass wool washed in Medium I to remove low-density lipid aggregates. The filtered suspension was centrifuged at 40 000xg for 90 min and the sediment resuspended in 55ml of Medium 2: 10mM Histidine, 0.6MKCl, pH 7.0. After 40 min incubation at 2 - 4°C the suspension was centrifuged at 15000xg for 20 min and the supernatant collected and recentrifuged at 78000xg for 60 min. This final sediment was resuspended in 20 ml of a buffer containing 10mM Histidine, pH7.4 and 30% Sucrose. Stock suspensions of SR vesicles (at approximately 25mg/ml) were stored for up to 4 days at 0°C . The calcium transport activity remained stable during this period and varied between 1,5 and 2.1 µmol Ca^{2+} transported / min/mg protein at 25°C.

2.2. Reconstitution of Sarcoplasmic Reticulum vesicles

The method used is essentially as described by Repke and coworkers (1976). Except as otherwise noted sarcoplasmic reticulum was solubilized at a protein concentration of 4mg/ml and a deoxycholate concentration of 0,55 mg per mg of protein. The solubilization step was performed at 0-4°C in the presence of a solubilization mixture containing 0,15M KCl, 20 mM Tris-Cl at pH 7.25, 2mM $MgCl_2$, ImMEDTA and 1mM dithiothreitol. The solubilized

mixture/...

mixture was centrifuged immediately for 1h at 176 000xg. Control sarcoplasmic reticulum was incubated in a similar solubilization mixture and the step of centrifugation omitted. All protein suspensions were made 0.4 M in sucrose before dialysis. Control and deoxycholate - solubilized sarcoplasmic reticulum were dialysed separately in 1 cm diameter dialysis bags which were previously boiled for 1h in 0,1M EDTA. Deoxycholate was removed from the solubilized protein by dialysis at 21 - 22°C for 21h against several changes of a medium containing 0.45MKCl, 20mM Tris-Cl at pH 7.25, 2mM Mg Cl_2, 1mM EDTA, 1mM dithiothrietol and 0,4M sucrose. Subsequent to dialysis, both the control and reconstituted sarcoplasmic reticulum preparations were centrifuged for 1h at 176000xg. The pellets were suspended in 0.4M sucrose and 20mM Tris-Cl at pH 7.25 and used immediately for assays. Dialysis of DOC from solibilized protein was monitored with (Carboxyl - C^{14}) - deoxycholate. The amount of protein solubilized was calculated from the amount of insoluble protein recovered in the pellet after the reaction mixture containing deoxycholate was centrifuged.

2.3. Acid inactivation of sarcoplasmic reticulum

Acid inactivation of calcium transport activity in isolated SR vesicles was followed at 37°C in a continuously stirred vessel. Inactivation was initiated by diluting stock suspensions of SR (18,5 mg protein/ml) with 40mM ammonium acetate buffer, pH 5.6 containing 1mM dithiothrietol to a final concentration of 2mg protein per ml. The pH of the suspension was maintained at 5.6 using a glass electrode and 0.1N HCl added from a Hamilton syringe. Inactivation was terminated by diluting the incubation medium 3 fold with ice-cold 100mM Tris-Cl buffer, pH 7.6 containing 35mM sucrose.

This method is a modification of the method described by Berman et al. (1977).

2.4./....

2.4. Calcium uptake assays

ATP -dependent calcium uptake activity was measured at 25°C in 50mM potassium phosphate, 0.12MKCl, 40mM histidine, pH 6.8, 0.5mM EGTA, 0.5mM $^{45}CaCl_2$ and 5mM Mg ATP. Calcium uptake was initiated by the addition of SR protein to a final concentration of 50 µg per ml. The initial rate of uptake was taken as the linear part of the slope of the calcium uptake plot based on four measurements taken between 0,5 s and 2 min. All samples were filtered through Type HA Millipore filters of 0.45 µ pore size. The filters were dissolved in Instagel scintillation cocktail (Packard) before being read in a Beckman LS-233 liquid scintillation spectrophotometer.
The method is essentially that described by Martonosi and Feretos (1964).

2.5. EGTA - induced Ca^{2+} release

Calcium release was measured after SR vesicles were loaded to a predetermined level in the presence of 50mM potassium phosphate. Ca^{2+} efflux was induced by the addition of an aliquot of 100mM EDTA to a final concentration of 3.3mM. The amount of Ca^{2+} remaining in the vesicles was determined by filtration as before.

2.6. Assay of ATPase activities

ATPase activity was measured by the NADH- coupled method as described by Horgan et al. (1972). Absorbance changes were measured on a Varian Techtron spectrophotometer at 30°C. Assays were performed on 2.57 ml samples at a final concentration of 20mM histidine at pH 6.8, 50mM KCl, 5mM Mg Cl_2, 0.5mM EGTA, 2.5mM phosphoenolpyruvate and 0.1mM NADH. Pyruvate kinase and lactate dehydrogenase were added to a final concentration of 8units/ml. The final concentration of SR protein was approximately 0.01mg/ml. The reaction was initiated by the addition of 1,0mM ATP and the change in absorbance per minute of basal ATPase

activity/...

activity recorded. Ca^{2+} -dependent ATPase activity was initiated by the addition of 0,5mM $CaCl_2$ and the change in absorbance for the (Ca^{2+}, Mg^{2+}) - ATPase recorded. The activity (µmol Pi released/mg protein/min) 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.

2.7. SDS -Polyacrylamide gel electrophoresis

2.7.1. Preparation of polyacrylamide gels

Polyacrylamide gels were prepared according to the method described by Weber and Osborn (1969) . Gel buffer was prepared to contain 7.8 g $NaH_2PO_4 \cdot H_2O$, 38.6g of $Na_2HPO_4 \cdot 7H_2O$ and 2g sodium dodecyl sulphate per litre. A 10% acrylamide solution was prepared by dissolving 22.2g of acrylamide and 0,6g of methylene bis-acrylamide in 100 ml double distilled water. Insoluble material was removed by filtration through Whatman No 1 filter paper and the solution kept in the dark at 4°C. For a typical run of 12 gels, 15ml of gel buffer was deaerated and mixed with 13,5 ml of the acrylamide solution. Subsequent to further deaeration, 1,5ml of freshly prepared ammonium persulphate solution (15mg / ml) and 45 µl of TEMED were added. After mixing, gels were prepared by filling clean glass tubes (10cm long and 6mm in diameter) with 2ml of the solution. Before the gel hardened a few drops of water were layered on top of the gel solution. An interface formed within 10 to 20 min upon hardening of the gel. Protein solutions were dissolved in phosphate buffer A containing 0.01M sodium phosphate, 0.1% SDS and 0.1% β mercapto-ethanol to a final concentration of 0.6 mg protein per ml. For each 10 µl of tracking dye (0.05% bromphenal blue in water), 1 drop of glycerol, 5 µl mercapto-ethanol and 50 µl of phosphate buffer A were mixed in a small test tube. 10 - 50 µl of the protein sample was added and after mixing the protein solutions were applied to the

gels/...

gels. The tubes were filled by carefully layering gel buffer diluted 1:1 on top of the samples. Electrophresis was performed at a constant current of 8mA per gel with the positive electrode in the lower chamber. Under our conditions the marker dye migrated through the gel in 6 to 8h.

After electrophoresis the gels were removed from the tubes by forcing water between the gel and glass wall by means of a syringe. Gels were stained with a staining solution containing 1.25 g coomassie brilliant blue in 454 ml of 50% methanol and 46ml glacial acetic acid. Staining was performed at room temperature for 2 - 4 h. After staining the gels were rinsed with distilled water prior to being electrophoretically destained in a solution containing 75ml acetic acid, 50ml methanol and 875ml water.

2.7.2. Recovery of protein from acrylamide gels

Protein was recovered from polyacrylamide gels according to the method described by Djondurov and Holtzer (1979). The stained bands containing protein were cut out of the gels using a razor blade and each put in a 4ml glass-teflon homogeniser. The gel slices were completely homogenised in 1ml of cold 10%TCA - 30% ethanol solution. The homogenates were transferred to tubes and centrifuged at 163000xg for 10 min. The supernatants were discarded and the same procedure repeated four more times. After the last centrifugation, the pellets of completely decolorised gels were treated with 1ml of 0.1N NaOH and shaken for 2h at 37°C, recentrifuged for 10 min at 23300xg and the supernatants carefully collected with Pasteur pipettes. It was possible to recover about 0,85ml of the original volume used. The recovered volumes were returned to 1ml with 0.1N NaOH and tested for protein.

2.8./...

2.8. Determination of tightly bound Ca^{2+} in SR membranes

Vesicles were reconstitued as described above. After concentration at 176000xg the vesicles were suspended in 20mM Imidazole buffer pH7.2 containing 80mMKCl and 2,5mM Mg Cl_2. An aliquot of isotopically labelled 100mM Ca Cl_2 was added to the protein to a final concentration of 5mM Ca Cl_2. The vesicles were equilibrated for 2h on ice and subsequently diluted 30fold into an efflux medium containing 20mM Imidazole at pH 7.2, 80mM KCl, 2.5mM Mg Cl_2, 1mM EGTA. 1ml aliquots of the efflux medium were filtered on 0,45 µ Millipore filters at given time intervals. Each filter was washed with 3 x 5ml aliquots of a washing buffer containing 20mM Imidazole at pH 7.25, 80mM KCl, 2.5mM Mg Cl_2, 0.066mM Ca Cl_2. The filters were dissolved in 200 µl deionised water and 5 ml Instagel before being read in a Beckman LS -233 Scintillation spectrophotometer.

2.9. Determination of the tightly-bound nucleotide content of SR membranes.

2.9.1. Enrichment of SR membranes with tightly bound nucleotides.

SR vesicles (1mg / ml) were enriched in nucleotide content by incubation at 30°C for 3 min in a medium containing 50mM histidine, pH 7.0, 50mM KCl, 5mM $MgCl_2$, 5mM EGTA, 4.7 mM $CaCl_2$ (free Ca^{2+} ions $\simeq$ 5 µM), 5mM PEP, 30 units/ml pyruvate kinase and 5mM ATP. The reaction was initiated by the addition of SR vesicles and the reaction terminated by incubation at 0-4°C. Loosely-bound nucleotides were removed from the SR membrane by passage through a Dowex-I column (2.9.2.).

2.9.2. Removal of loosely-bound nucleotides from SR membranes.

Loosely-bound nucleotides were removed from SR membranes by passage over a column of the anion exchange resin, Dowex-I x 8 -200.

Dowex/....

Dowex was regenerated by stirring 100 ml of Dowex I with 2ℓ of 1M NaOH for 15 min at room temperature, following which the suspension was allowed to settle, the NaOH decanted and the suspension washed 10 times with 1,5ℓ of doubled-distilled deionised water. After the final wash 200 ml IN formic acid was added to the resin which was stirred for 15 min at room temperature. The acid was decanted and the resin washed 10 times with 1,5ℓ deionised water until neutral. Imidazole 10mM, pH 7.4 (1ℓ) was added to the neutral resin, the suspension stirred for 15 min, the resin allowed to settle and the buffer decanted. The resin was stored in 500ml 10mM Imidazole pH7.4 for up to one week at 0-4°C.

Columns (7cm) of Dowex were formed in Pasteur pipettes the outlets of which had been loosely packed with glass wool. Disposable 5ml plastic syringes were attached to the tops of the columns as reservoirs and the columns equilibrated with 10ml of 10mM Imidazole buffer, pH7.4 containing 0,25M sucrose. The whole procedure was performed at 0-4°C.

Samples (5ml) were passaged through the columns followed by 2ml of 10mM imidazole, pH7.4 and 0,25M sucrose. Samples were collected, concentrated at 105 000 x g and resuspended in 10mM Imidazole, pH 7.4 and 0.25M sucrose. Total nucleotide content was determined by taking 50 µl SR protein into 15ml Instagel (Packard) and the radioactivity read in a Beckman LS 233 scintillation spectrophotometer.

2.10. Determination of protein concentrations.

Protein concentrations were determined according to the method of Lowry et al. (1951). Protein samples (20µl containing 15 - 40µg protein) were mixed with 50µl 10% sodium deoxycholate to a final volume of 200 µl in distilled water. Alkaline copper sulphate solution (1ml) containing 0.015% cupric sulphate, 0.05% sodium tartrate, 2% sodium carbonate and 0,1M sodium hydroxide was added to the protein suspension and the mixture allowed to stand for 10min. Folin - Ciocalteu reagent (100 µl) was added with rigorous mixing and the colour allowed to develop for 30 min. Protein concentrations were determined from changes in the

optical/...

optical density at 750nm using bovine serum albumin standards.

3.0. RESULTS

3. RESULTS

3.1. Solubilization and reconstitution of isolated sarcoplasmic reticulum vesicles.

SR vesicles were reconstituted from the supernatant obtained after centrifugation of SR solubilized in deoxycholate (see 'Materials and Methods'). Using the standard procedure for reconstituion, as described by Repke et al. (1976), 40% of the SR protein was solubilized at a deoxycholate concentration of 0,55 mg DOC/mg protein compared to a value of 73% obtained by Repke and coworkers. The recovery of reconstituted vesicles expressed as a percentage of the total amount of protein solubilized was 10.4% at 0.55 mg DOC/mg protein.
The time course for calcium transport in reconstituted vesicles, compared with the transport activity measured in original SR vesicles is shown in Figure 1. The transport activity, as measured in reconstituted vesicles and expressed as a ratio of the activity measured in original vesicles was 10%, compared to a figure of 87% obtained by Repke and collaborators.

3.1.1. The effect of sucrose on the reconstitution of SR membranes.

Deoxycholate - solubilized SR protein was dialysed in the presence and absence of 0.4M sucrose to determine the effect of the presence of sucrose during slow dialysis on the reconstitution of Ca^{2+} transport activity. The data for ATP-dependent calcium transport activity in vesicles reconstituted in the presence and absence of sucrose are presented in Figure 2. In agreement with the results of Repke et al. (1976), we found that the presence of sucrose during dialysis was beneficial but not essential for the reconstitution of Ca^{2+} transport activity in DOC-solubilized SR.

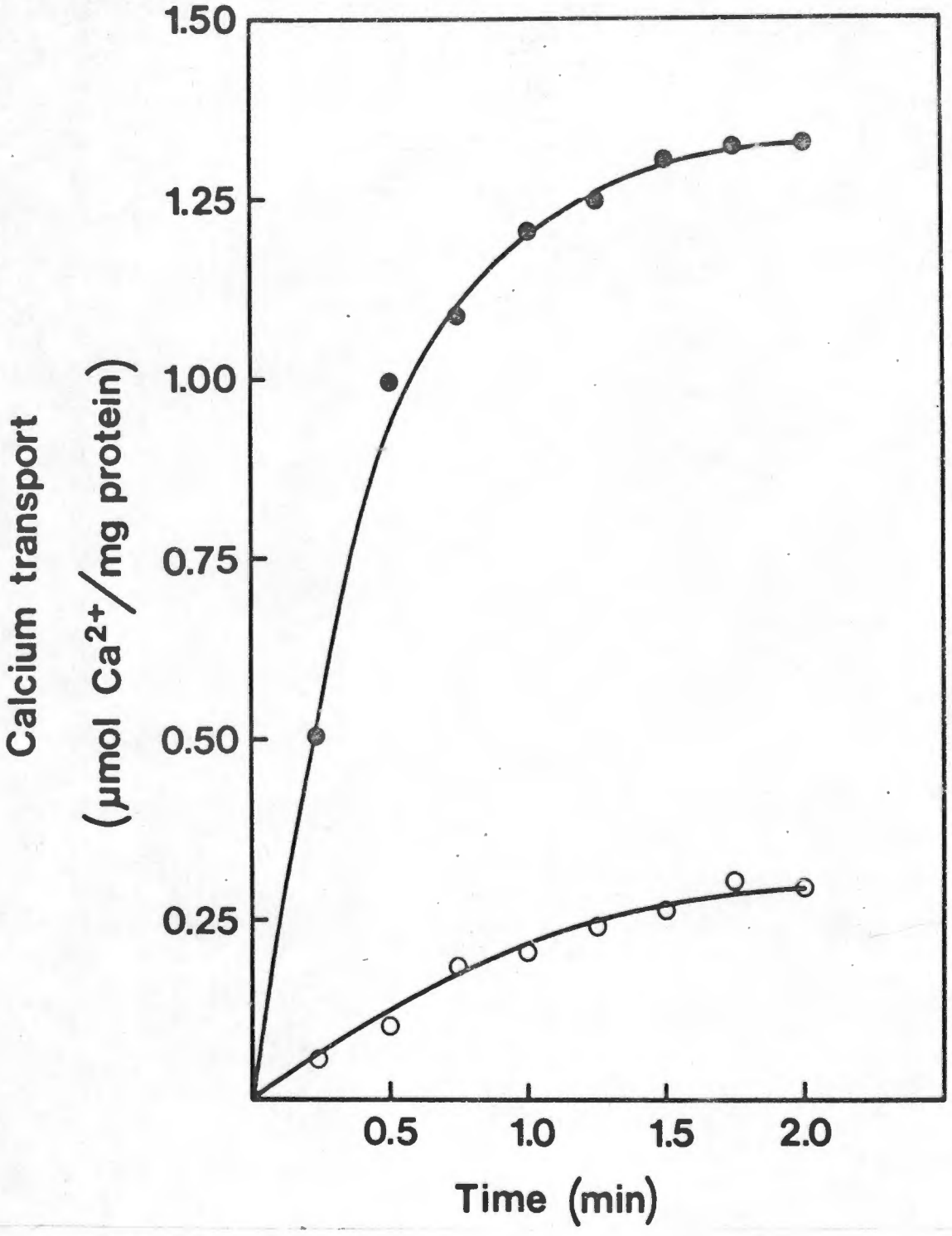

FIGURE 1 Time course of calcium transport activity in original (●) and reconsituted SR vesicles (○).

Vesicles were reconstituted from the 176 000 x g supernatant of DOC - solubilized protein. Protein was solubilized at a deoxycholate concentration of 0.55 mg DOC/mg protein and dialysed at 21°C in the presence of sucrose. Vesicles were loaded in the presence of 50mM potassium phosphate as described under 'Materials and Methods'.

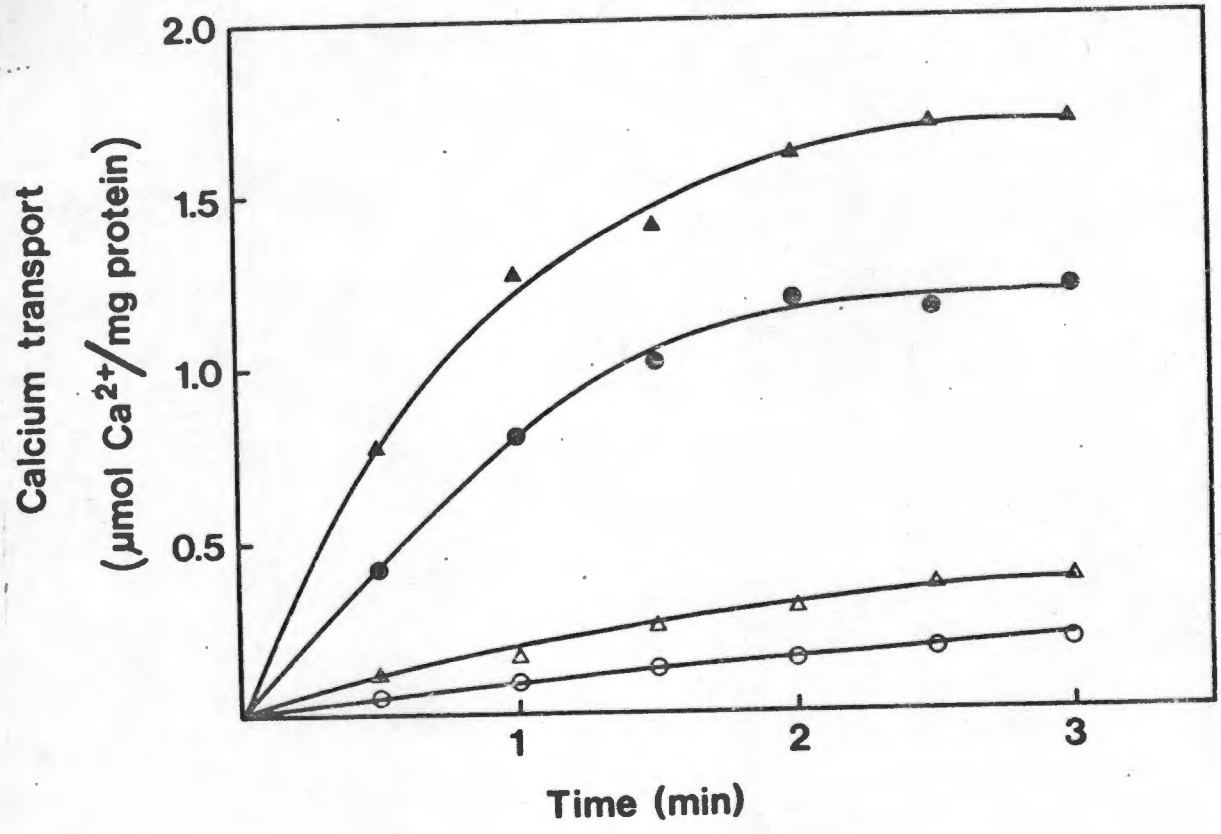

FIGURE 2 The effect of sucrose during dialysis on calcium transport in original and reconstituted vesicles.

Vesicles were solubilized in the presence of 0.15MKCl, 20mMTris- Cl, pH 7.25, 2mM $MgCl_2$, 1mM EDTA and 1mM dithiotrietol at a protein concentration of 4mg protein/ml and deoxycholate concentration of 0.55mg DOC/mg protein. Control SR was diluted with the above medium to a concentration of 4mg protein/ml and the deoxycholate omitted. Both the control and the solubilized protein were dialysed in the presence and absence of 0.4M sucrose at 21°C

KEY
▲—▲ control vesicles (+ sucrose)
●—● control vesicles (- sucrose)
△—△ reconstituted vesicles (+ sucrose)
○—○ reconstituted vesicles (- sucrose)

3.1.2. The effect of oxalate on calcium transport in reconstituted vesicles.

Racker and collaborators (Racker, 1972; Racker and Eytan, 1973) reconstitute particulate Ca - ATPase liposomes in the presence of a buffer containing the calcium sequestering anions, oxalate or phosphate, which become trapped inside the reconstituted vesicles.

In order to determine the effect of oxalate during dialysis on the quality of the reconstituted vesicles, the solubilization and dialysis buffers as described by Repke et al. (1976; Materials and Methods) were modified to contain 20mM Tris-oxalate, pH 7.25.

The effect measured (Fig 3) is contrary to that found by Racker and coworkers since vesicles reconstituted in the absence of oxalate displayed higher initial rates of uptake than those reconstituted in the presence of oxalate. The reasons for this discrepancy are not obvious.

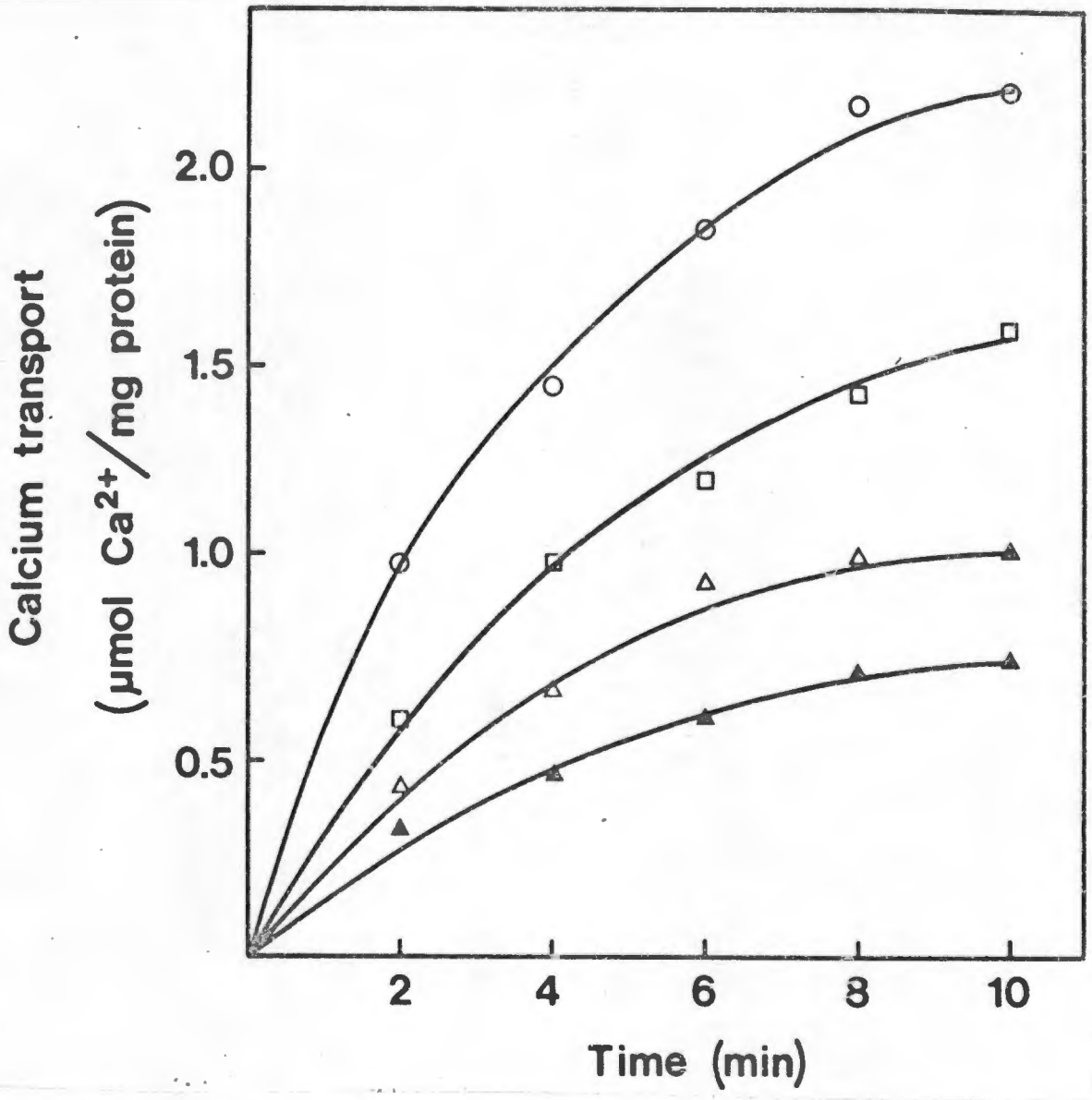

FIGURE 3 The effect of oxalate on calcium transport in reconstituted vesicles.

DOC-solubilized SR protein (o.55mg DOC/mg protein) was dialysed in the presence and absence of 20mM Tris-oxalate, pH 7.25. Vesicles reconstituted in the presence of oxalate were dialysed against a buffer containing 20mM Tris-oxalate, pH7.25, 0.4M sucrose, 0.45MKCl, 2mM $MgCl_2$, 1mM EDTA and 1mM dithiothrietol. Control vesicles and vesicle reconstituted in the absence oxalate were dialysed as described under 'Materials and Methods'. Original SR vesicles were kept on ice in o.4M sucrose and 20mM Tris-cl, pH 7.25.

KEY
- o—o Original SR
- □—□ Control SR
- △—△ Vesicles reconstituted in the absence of oxalate
- ▲—▲ Vesicles reconstituted in the presence of oxalate

3.1.3. The effects of varying deoxycholate concentrations during solubilization on reconstitution.

Sarcoplasmic reticulum vesicles are not completely solubilized at 0.55 mg DOC/mg protein. Meissner and Fleischer (1974) claim 70 - 80% solubilization at 0.48 mg DOC/mg protein; under our conditions we solubilized 40% of the protein at 0.55 mg DOC/mg protein as measured by protein determinations of the 176 000xg pellet. Three deoxycholate concentrations i.e. 0.55, 0.66 and 0,77 mgDOC/mg protein were employed to determine the optimal deoxycholate concentration for our conditions. Increased amounts of protein were solubilized with increasing deoxycholate concentrations, although optimal transport activity was obtained at 0.66 mg DOC/mg protein (Table 1). Deoxycholate, at a concentration of 0,77 mg DOC/mg protein solubilized about 60% of the protein but calcium transport activity at that concentration was approximately half that obtained at 0,66 mg DOC/mg protein.

TABLE 1 Effect of increasing deoxycholate concentrations on the solubilization of SR proteins.

DOC concentration (mg DOC/mg protein)	Protein solubilized (% of total)	Calcium uptake umol/min/mg
0,55	40	0,20
0,66	51	0,45
0,77	62	0,23

SR protein was solubilized in the absence of sucrose in a buffer containing 0.15 MKCl, 20mM Tris-Cl, pH 7.25, 2mM $MgCl_2$, 1mM EDTA and 1mM dithiothrietol. Deoxycholate was added from a 10% solution to final concentrations of 0,55, 0,66 and 077 mg DOC/mg protein. The amount of protein solubilized was determined by assaying the 176 000 x g supernatant as well as the 176 000 x g pellet for protein. Deoxycholate was removed from the solubilized protein by slow dialysis (Materials and Methods).

3.1.4. Rate of dialysis of DOC from solutions of DOC - solubilized protein.

The rate of dialysis of deoxycholate from detergent solubilized vesicles was monitored with (Carboxyl - C^{14})-deoxycholate. About 50% of the detergent dialysed from the dialysis bag within the first 2 - 3 h of dialysis (Fig 4). The final amount of deoxycholate associated with the vesicles after 21h of dialysis at room temperature was found to be approximately 0.15 mg DOC/mg protein.
In other words an amount of DOC equivalent to 0.15 mg/mg protein appeared to be resistant to removal by dialysis. This residual DOC in reconstituted SR preparations has not been previously commented upon.

3.1.5. Electron - microscopy of original and reconstituted vesicles.

In order to detect any morphological differences in original and reconstituted vesicles, vesicles were fixed in 1% OsO_4 and subjected to electron microscopy.
Reconstituted and original SR vesicles appear morphologically similar as viewed by electron microscopy (Fig. 5). However, in agreement with previous results (Meissner and Fleischer, 1974; Repke et al. 1976) the reconstituted vesicles appear larger than original vesicles.

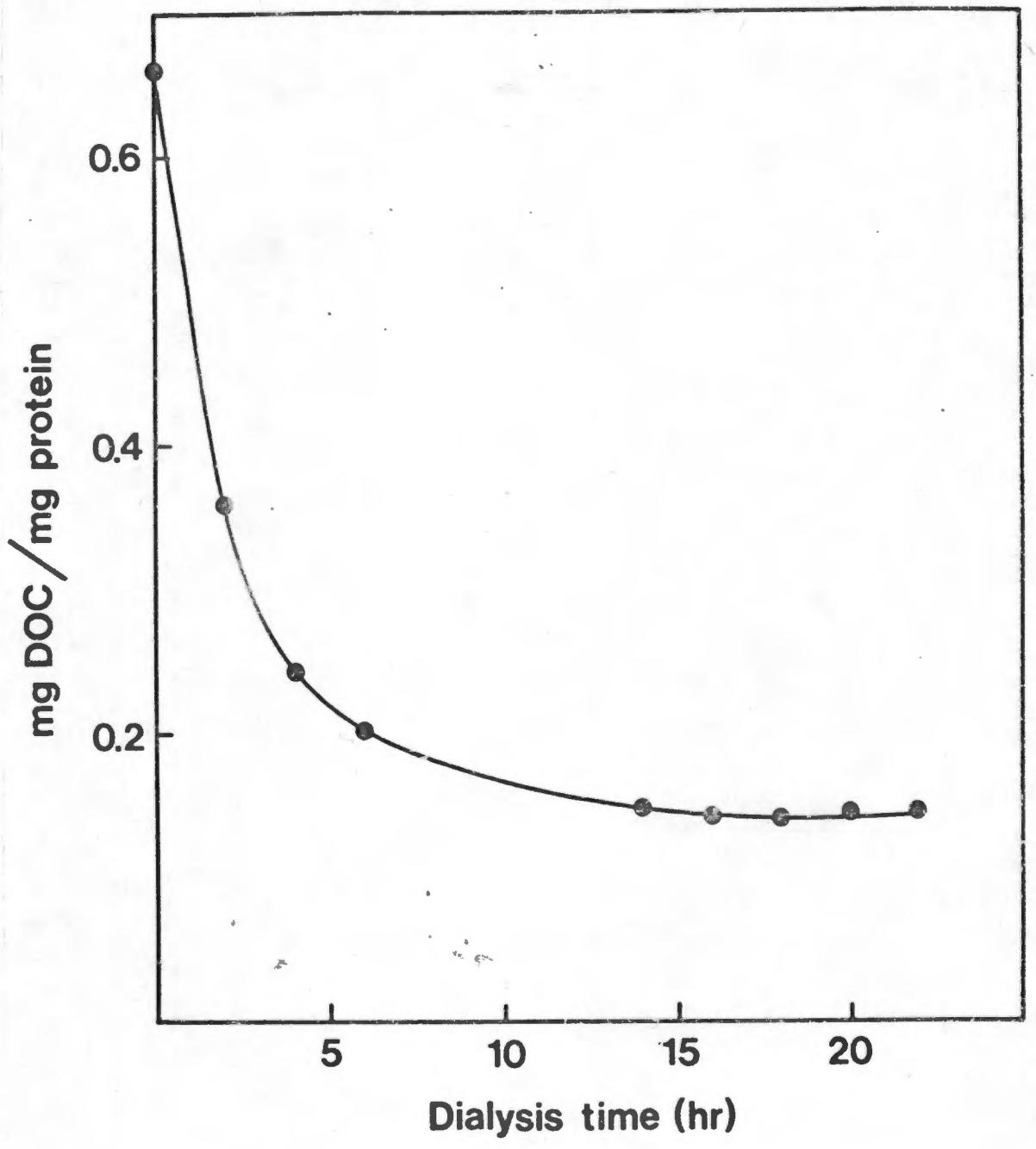

FIGURE 4. Dialysis of deoxycholate from solubilized protein.

The dialysis of deoxycholate from solubilized protein was monitored with (carboxyl-C^{14})-deoxycholate. Aliquots of solubilized protein were taken at intervals during the dialysis procedure and the radioactivity measured in the presence of the scintillant Instagel (Packard Instruments) in a Beckman LS - 233 liquid scintillation spectrophotometer.

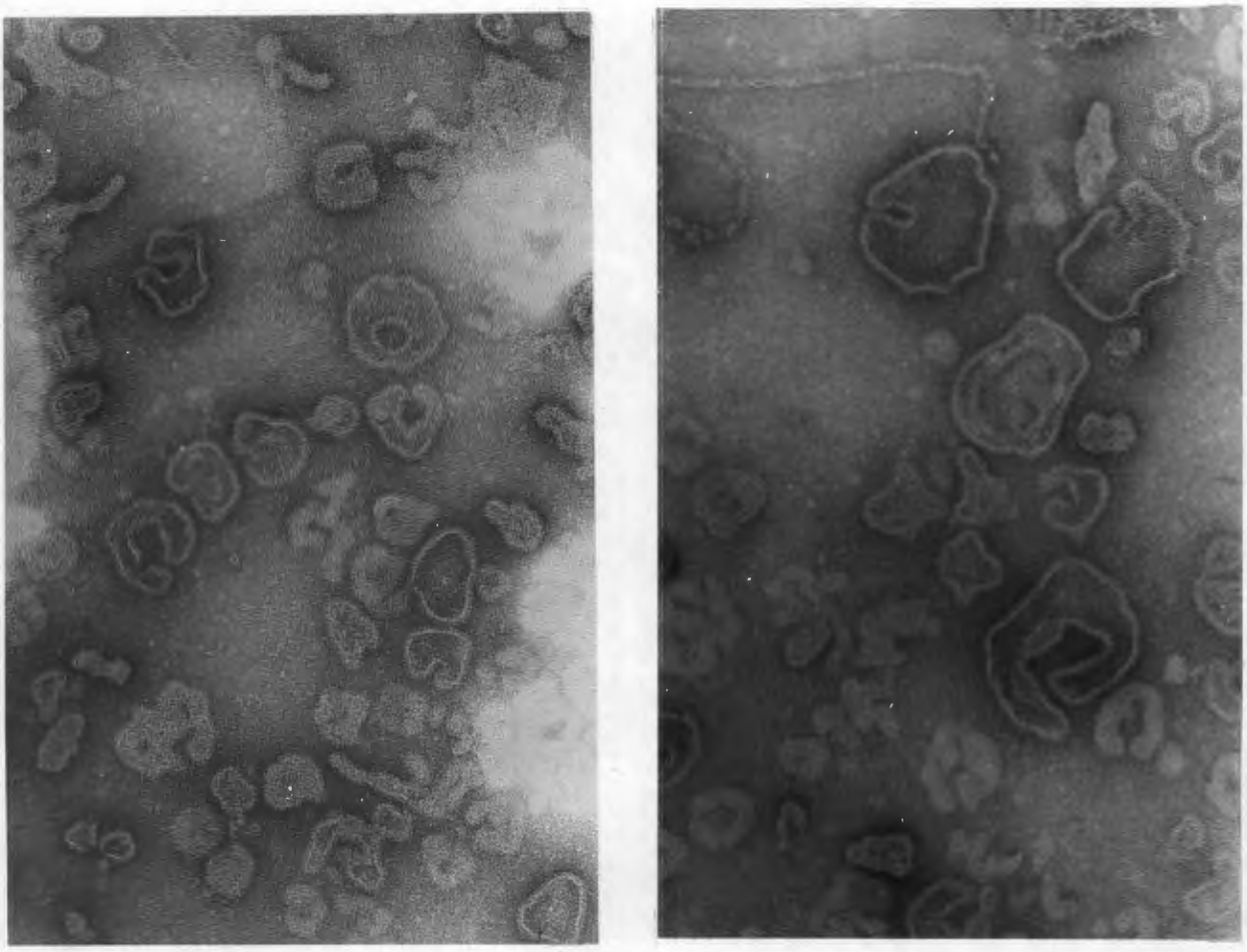

FIGURE 5 Electron micrographs of original (left) and reconstituted (right) vesicles (120 000 x magnification).

SR protein was solubilized with 0.66 mg DOC/mg and dialysed for 21h against several changes of a medium containing 0.45 MKCl, 20mM Tris-Cl, pH 7.25, 2mM $MgCl_2$, 1mMEDTA, 1mM dithiothrietol and o.4M sucrose. Reconstituted vesicles were concentrated at 176 000 x g for 1h, resuspended in 0.4M sucrose and 20mM Tris-Cl, pH 7.25 and immediately fixed in 1% OsO_4 .

3.2. Reconstitution of Acid-inactivated sarcoplasmic Reticulum.

3.2.1. The effect of acid exposure on Ca^{2+} transport activity in native SR.

The time course for acid inactivation of calcium transport in sarcoplasmic reticulum was followed at 37°C and pH 5.65. Calcium transport activity decayed exponentially with increasing incubation times (Fig.6). 90% inactivation was achieved after 8 min under our conditions. These data are similar to those reported previously by Berman et. al. (1977).

3.2.2. Reconstitution of original and acid-inactivated SR.

Acid inactivated SR vesicles do not regain calcium transport activity if stored at room temperature or on ice for up to 72h (Berman et al. 1977). The possible reversal of inactivation by reconstitution of acid inactivated vesicles was studied. The results are shown in Fig 7. Original SR vesicles, subjected to DOC - solubilization and reconstitution regained approximately 65% of their original calcium transport activity, calculated on the basis of µmol Ca^{2+} transported per mg of SR protein. SR vesicles which had been inactivated by 90% by acid treatment regained approximately 27% of their original activity and 40% of the activity of reconstituted control vesicles.
The recovery of total protein in the pellet, sedimented at 176000 x g for 60 min from control and reconstituted vesicles was 9,25 mg and 0,92 mg respectively

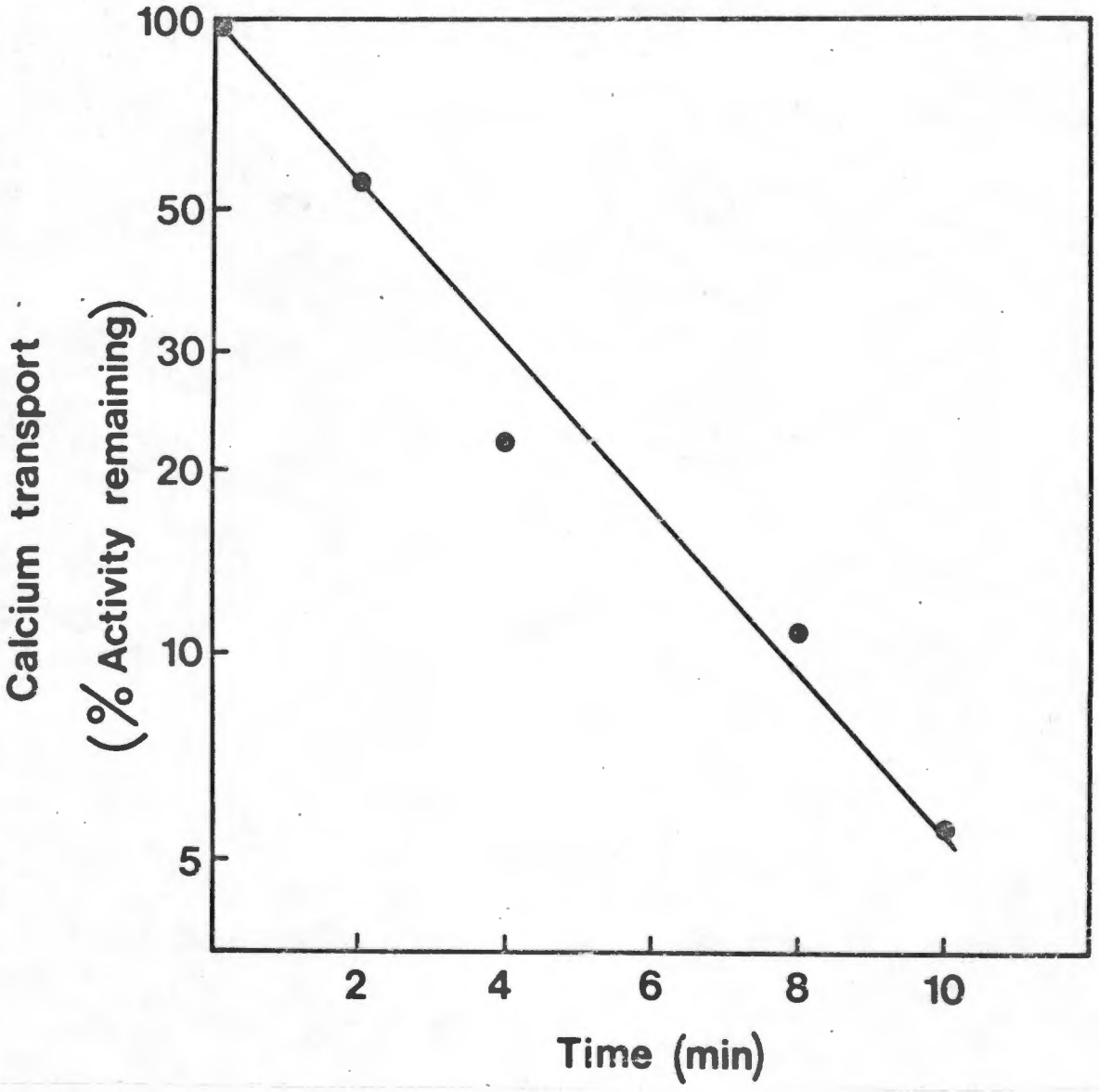

FIGURE 6 The effect of mild acid exposure on the calcium transport activity of isolated SR vesicles.

Acid inactivation of calcium transport activity in isolated SR vesicles was followed at 37°C in a continuously stirred vessel. Inactivation was initiated by diluting stock suspensions of SR protein with 40mM ammonium acelate buffer, pH 5.65 containing 1mM dithiothrietol. Inactivation was terminated by diluting the incubation medium 3 fold into 100mM Tris-Cl, pH 7.60 and 35mM sucrose

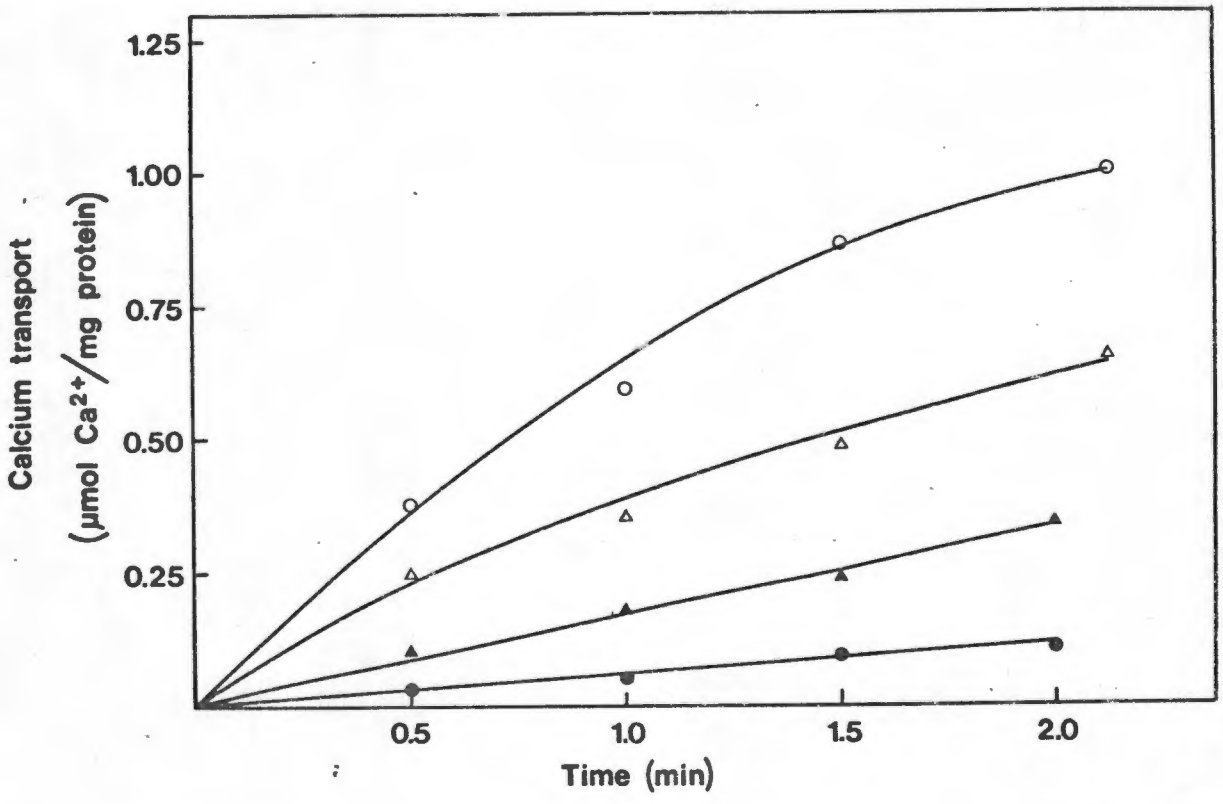

FIGURE 7 Calcium transport activity of reconstituted-control(△)and reconstituted acid inactivated SR(▲).

SR protein was acid inactivated as described in the legend to Fig. 6. Subsequent to inactivation, vesicles were concentrated at 30 000r.p.m. in a 30 Type Rotor (Beckman) for 30 min. and resuspended in solubilization mixture (legend Figure 2; Materials and Methods) to a concentration of 4mg protein/ml. Membrane suspensions were solubilized with 0.55 mg DOC/mg protein and dialysed for 21h as described above (legend fig. 3). 'Reconstituted control vesicles' refers to vesicles reconstituted from non-acid inactivated (original) SR. 'Reconstituted acid inactivated vesicles' refers to vesicles reconstituted from acid inactivated SR.

O CONTROL SR

● ACID-INACTIVATED SR

3.2.3. SDS gel electrophoretic analysis of original, control and reconstituted SR.

Original SR vesicles contain predominantly (≃ 80%) the 102 000 dalton ATPase, M-55 (55 000 dalton) and calsequestrin (45 000 dalton) proteins previously described (Martonosi, 1969; Mac Lennan and Wong, 1971; Mac Lennan et al. 1972). In addition a faint unidentified species migrating between the ATPase and M55 calcium binding protein (CBP) is evident. Phosphorylase b (MW. = 90 000) is known to comigrate with the ATPase (Figures 8 and 9). This species appears to be lost in control and reconstituted vesicles. While the control vesicles retain amounts of calsequestrin and calcium binding protein comparable to original SR vesicles, DOC - solubilized vesicles appear to lose calsequestrin during reconstitution and retain most of the calcium binding protein.

Table 2 summarises the value of total SR protein corresponding to each electrophoretic fraction as determined by densitometry and protein extraction (Materials and Methods).

These data show an apparent enrichment of ATPase content in reconstituted vesicles with a corresponding loss of the 45K (Calsequestrin) fraction which cannot be attributed to the effects of acid inactivation and subsequent slow dialysis since both control vesicles show retention of the 55K and 45K fractions. Loss of the 45K and retention of the 55K fraction was previously shown by Meissner and Fleischer (1973) and Repke et al. (1976).

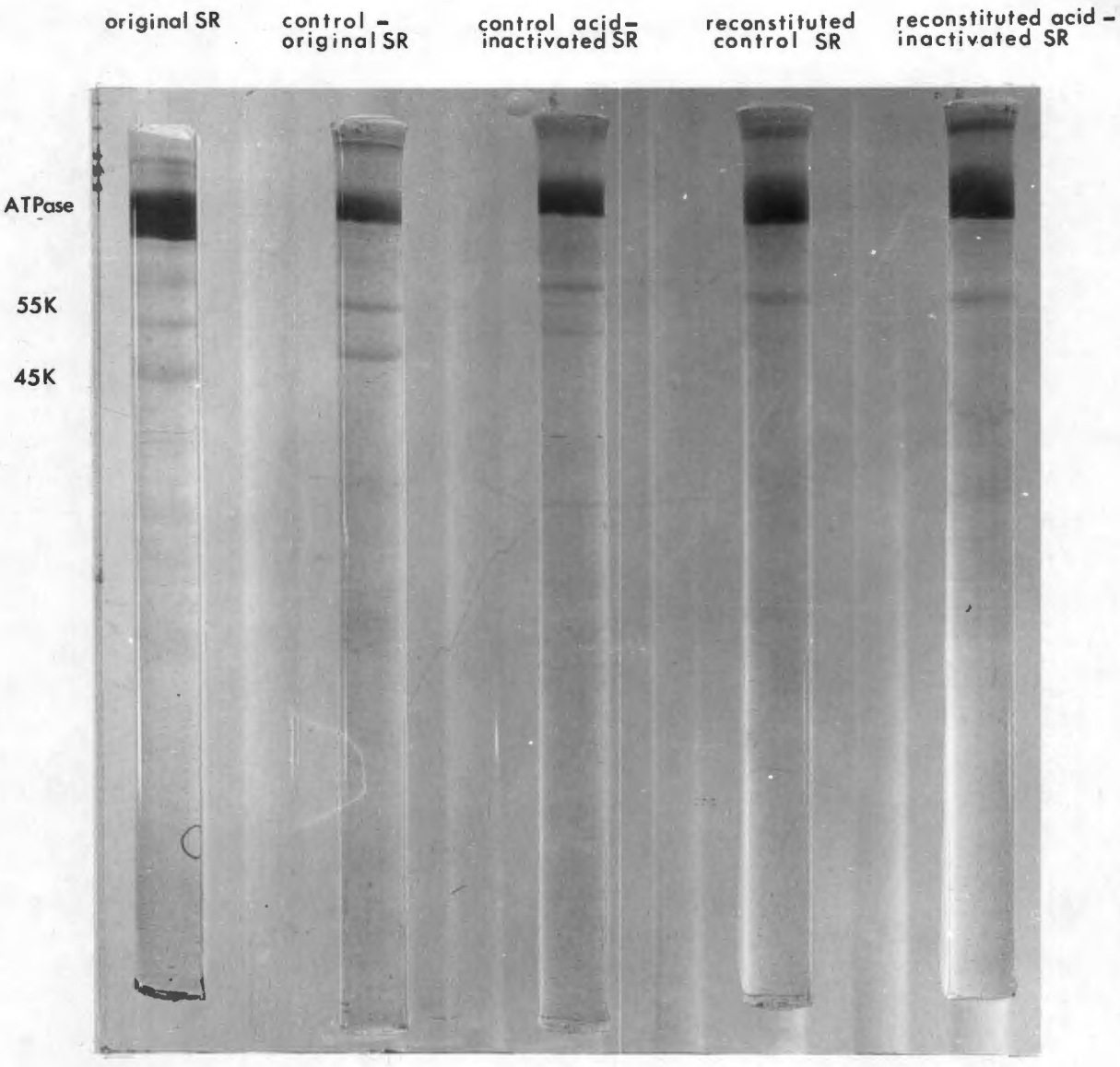

FIGURE 8 Electrophoretic separation of original, control and reconstituted vesicles.

Protein samples were dissolved in phosphate buffer containing 0.01M sodium phosphate, pH 7.0, 0.1% SDS and 0,1% β mercapto-ethanol to a final concentration of 0.6mg protein/ml. Gels were prepared as described under 'Materials and Methods' and electrophoresed at a current of 8mA per gel.

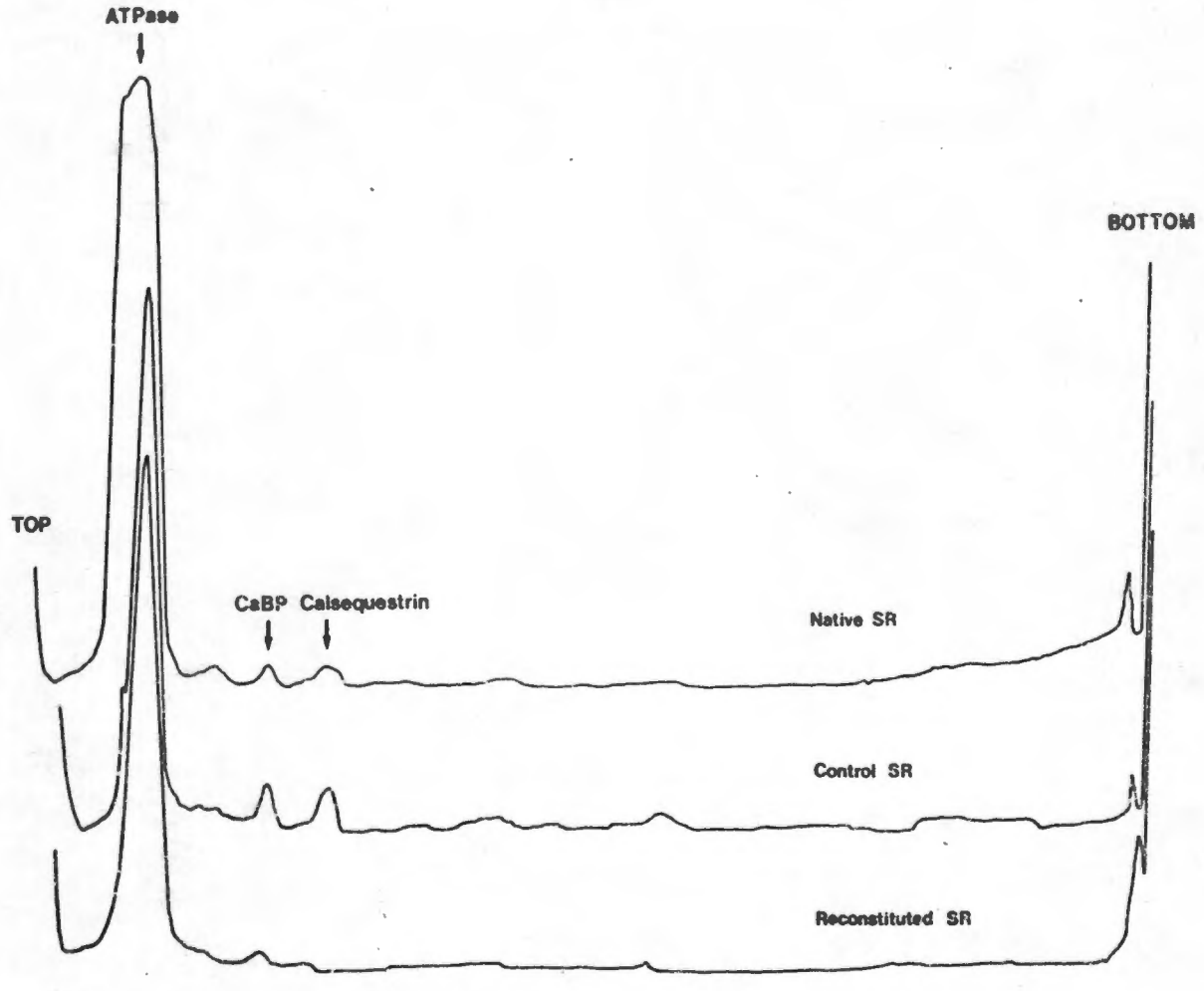

FIGURE 9 Densitometric scans of Coomassie Blue - stained SDS gels.

Gels were extruded from the glass tubes and stained with a solution containing 1.25 g Coomassie Brilliant Blue in 454 ml of 50% methanol and 46ml glacial acetic acid as described in 'Materials and Methods'. Gels were electrophoretically destained for 2h and the absorption profiles measured at 600nm .

TABLE 2. Relative fractions of SR proteins determined by densitometry and protein extraction.

Protein band		Original SR	Control-original SR	Control Acid-inactivated SR	Reconstituted Control SR	Reconstituted acid-inactivated SR
ATPase	-a	0,77	0,75	0,76	0,92	0,91
	-b	0,68	0,67	0,69	0,84	0,82
UNIDENTIFIED	-a	0,10	-	-	-	-
	-b	0,09	-	-	-	-
CALCIUM BINDING PROTEIN (M55)	-a	0,06	0,11	0,11	0,08	0,09
	-b	0,10	0,13	0,14	0,16	0,18
CALSEQUESTRIN (M45)	-a	0,07	0,14	0,13	-	-
	-b	0,13	0,20	0,17	-	-

a Fractional value determined by densitometry.

b Fractional value determined by protein extraction.

1. 'Original SR' refers to untreated SR stored at 0-4°C.
2. 'Control original SR' refers to original SR dialysed at room temperature without DOC - or acid-treatment.
3. 'Control acid-inactivated' SR refers acid inactivated SR dialysed at room temperature without DOC treatment.
4. 'Reconstituted control SR' refers to vesicles reconstituted from non-acid inactivated SR.
5. 'Reconstituted acid inactivated SR' refers to vesicles reconstituted from acid inactivated SR.

3.2.4. The effect of varying deoxycholate concentrations on calcium transport activity in reconstituted acid inactivated vesicles.

Figure 10 shows the effect of increasing deoxycholate concentrations on the calcium transport activity in reconstituted acid inactivated vesicles. In agreement with the results obtained for vesicles reconstituted from non-acid treated SR (Table 1), maximal transport activity was obtained from vesicles reconstituted from protein solubilized at a deoxycholate concentration of 0,66 mg DOC/mg protein.

3.2.5. Properties of Reconstituted Acid-treated SR vesicles

3.2.5.1. Comparison of the initial rates of calcium transport in original, control and reconstituted vesicles.

Original, control and reconstituted vesicles were compared with respect to their initial rates of Ca^{2+} transport within the first 30 minutes after concentration at 176 000 x g following slow dialysis at 21°C. The transport activity in reconstituted SR vesicles depended on the pretreatment and varied between 0,13 and 0,48 µmol Ca^{2+} transported/min/mg protein for reconstituted control vesicles and 0,075 and 0,440 µmol Ca^{2+} transported/min/mgprotein for reconstituted acid inactivated SR vesicles. The transport activity of the control vesicles also showed considerable variation (Table 3).

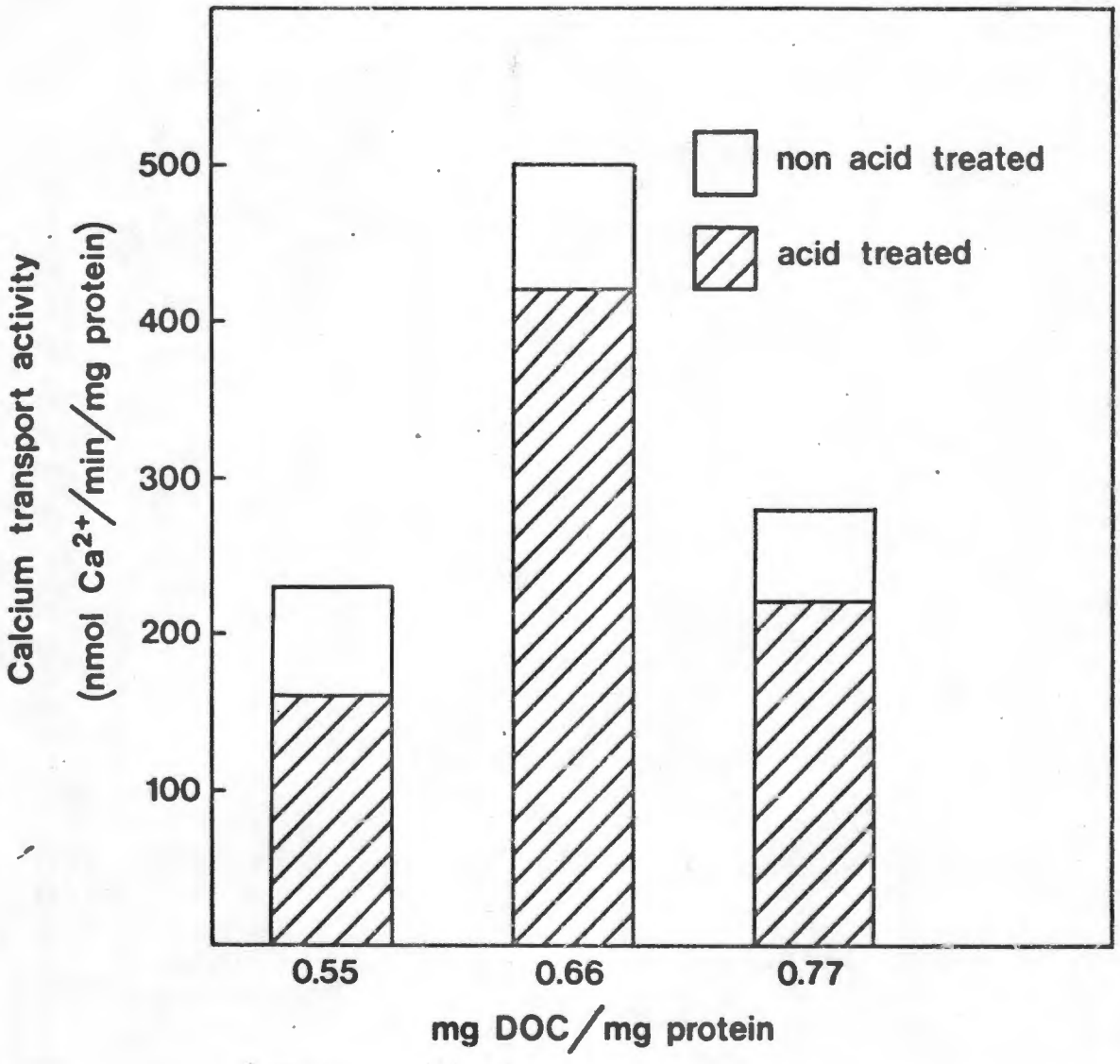

FIGURE 10 The effect of increasing deoxycholate concentrations on the reconstitution of acid inactivated SR.

Acid inactivated and original SR were concentrated at 30 000 r.p.m. (Legend fig. 7) and subsequently solubilized at 0,55 0,66 and 0,77 mg DOC/mg protein. Deoxycholate was removed from solubilized protein by slow dialysis at room temperature for 21h.

TABLE 3. Initial rates of Ca^{2+} transport activity in reconstituted, control and original SR Calcium transport activity is expressed as µmol Ca^{2+} transported/min/mg protein.

EXPT. NO	RECONSTITUTED CONTROL SR	RECONSTITUTED ACID-INACTIVATED	CONTROL ORIGINAL SR	CONTROL ACID INACTIVATED SR	ORIGINAL SR
1	0,48	0,44	0,76	0,090	1,50
2	0,34	0,27	0,90	0,093	2,00
3	0,14	0,075	0,29	0,035	1,57
4	0,21	0,115	0,46	0,082	2,12
5	0,28	0,174	0,69	0,04	1,52

The initial rates of transport were determined from the linear part of an uptake plot based on 4 values between 0,5 sec and 2 min. Calcium transport studies were performed at 25°C at a protein concentration of 50 µg/µl in a medium containing 50mM KCl, 20mM histidine pH 6,8, 0,5mM EGTA, 0.5mM $CaCl_2$ and 50mM potassium phosphate. Calcium transport was terminated by filtration through 0,45 µ Millipore filters.

3.2.5.2. A comparison of the initial rates of Ca^{2+} transport activity, ATPase activity and coupling ratios in SR vesicles.

ATPase dependent Ca^{2+} transport activity, ATPase activity and Ca:ATPase coupling ratios of original, control and reconstituted vesicles are summarised in Table 4. ATPase activity in control SR was elevated 4 - 6 fold above the activity in freshly isolated original SR vesicles. In contrast the ATPase activity of reconstituted vesicles declined to a value of between 11 and 33% of the value measured in freshly isolated original SR. ATP dependent Ca^{2+} transport activity in the reconstituted vesicles were of similar order as ATPase activity leading to higher Ca:ATPase coupling ratios in reconstituted vesicles. The decreased ATPase activity of the reconstituted vesicles is probably due to the prolonged exposure of the solubilized (Ca^{2+}, Mg^{2+}) ATPase to solubilizing concentrations of DOC.

TABLE 4. Initial rates of Ca^{2+} transport activity, ATPase activity and coupling ratios in SR vesicles.

VESICLE TYPE	Ca^{2+} Transport Activity (umol/min/mg protein)	ATPase Activity (umol/min/mg protein)	Ca ATPase ratio.
ORIGINAL SR	2.0	1.18	1,7
RECONSTITUTED CONTROL SR	0,33	0,29	1,1
RECONSTITUTED ACID-INACTIVATED SR	0,27	0,22	1,2
CONTROL ORIGINAL SR	1,50	6,43	0,23
CONTROL ACID-INACTIVATED SR	0,18	7,41	0,02

Vesicles were reconstituted, concentrated and resuspended in 0.4M sucrose and 20mMTris-Cl, pH7.25. Calcium transport activity was determined within 30min of resuspension as described in the legend to Table 3. ATPase activities were determined immediately afterwards by the NADH - coupled method (see Materials and Methods).

3.2.5.3. The effects of the calcium ionophore A23187 on Ca^{2+} - ATPase activity of reconstituted acid inactivated SR vesicles.

The effect of the calcium specific ionophore, A23187, on ATPase activity was investigated to determine whether the decreased ATPase activity observed in reconstituted vesicles was due to a tight coupling of Ca^{2+} transport activity to ATPase activity. Basal, extra-splitting and ionophoric-stimulated rates of ATP hydrolysis are shown in Table 5. Although the Mg^{2+} dependent ATPase activity in reconstituted vesicles also shows a degree of inhibition they are of the same order of activity as measured in control vesicles. The Ca^{2+} -dependent ATPase activity of reconstituted vesicles shows considerable inhibition compared to the Ca^{2+} -dependent ATPase activity of the control vesicles in which there is 3-fold elevation of activity above that of freshly isolated original SR vesicles.
The ionophore-stimulated Ca^{2+} -dependent ATPase activity in reconstituted vesicles was 1,5 times the non-ionophoric activity, compared to ionophore-stimulation of 1,8 and 1,1 to 1,3 in original and control vesicles respectively.
Even though the ionophoric stimulation of Ca^{2+}-dependent ATPase activity in reconstituted vesicles is of the same order as that observed in control vesicles, the total ATPase activity is decreased which suggests that inhibition of the (Ca^{2+},Mg^{2+}) ATPase has occured.

TABLE 5. Comparison of basal, extra-splitting and ionophoric - stimulated rates of ATP hydrolysis in original, control and reconstituted vesicles.

VESICLE TYPE	ATPase activity (umol/min/mg protein)			
	Mg^{2+}-dependent activity	Ca^{2+}-dependent activity	+A 23187	(Mg^{2+},Ca^{2+}) ATPase
ORIGINAL	0,276	1,43	2,65	1,15
RECONSTITUTED CONTROL SR	0,140	0,32	0,46	0,18
RECONSTITUTED ACID-INACTIVATED SR	0,114	0,23	0,34	0,112
CONTROL ORIGINAL SR	0,198	4.24	5,50	4,04
CONTROL ACID-INACTIVATED SR	0,171	4,79	4,94	4,62

ATPase activity assays were performed at 30°C in a medium containing 20mM histidine, pH 6.8, 50mMKCl, 5mM $MgCl_2$, 0,5mM EGTA, 2,5mM phosphoenolpyruvate, 0,1mM NADH and 8 units/ml (each) of pyruvate kinase and lactate dehydrogenase. SR protein was added to a final concentration of 0,01 mg/nl and the reaction initiated by the addition of 1,0mM ATP (see Materials and Methods). Ionophoric stimulation of ATPase activity was achieved by the addition of the calcium specific ionophore A23187 to a final concentration of 40 μM.

3.2.5.4. EGTA - induced efflux of Ca^{2+}

The EGTA - induced efflux of Ca^{2+} from control and reconstituted vesicles is shown in Figure 11. The Ca^{2+} efflux was biphasic and was initiated by the addition of 3,3mM EGTA to vesicles which had been actively loaded for 10 minutes in the presence of ATP. The initial rates of Ca^{2+} transport activity, the final amount accumulated after 10 minutes, the initial rates of efflux and the final amount remaining in the vesicles after 6 minutes of efflux are presented in Table 6. The data presented in Figure 11 and Table 6 indicate that reconstituted vesicles do not efflux to the same extent as do control vesicles. This is in agreement with the results of Repke et al. (1976) who showed EGTA - induced calcium release to be slower in reconstituted vesicles than in control vesicles.
Control Vesicles effluxed to 40 - 50% of the total amount of Ca^{2+} present at the initiation of efflux compared to 60 - 70% in the reconstituted vesicles.

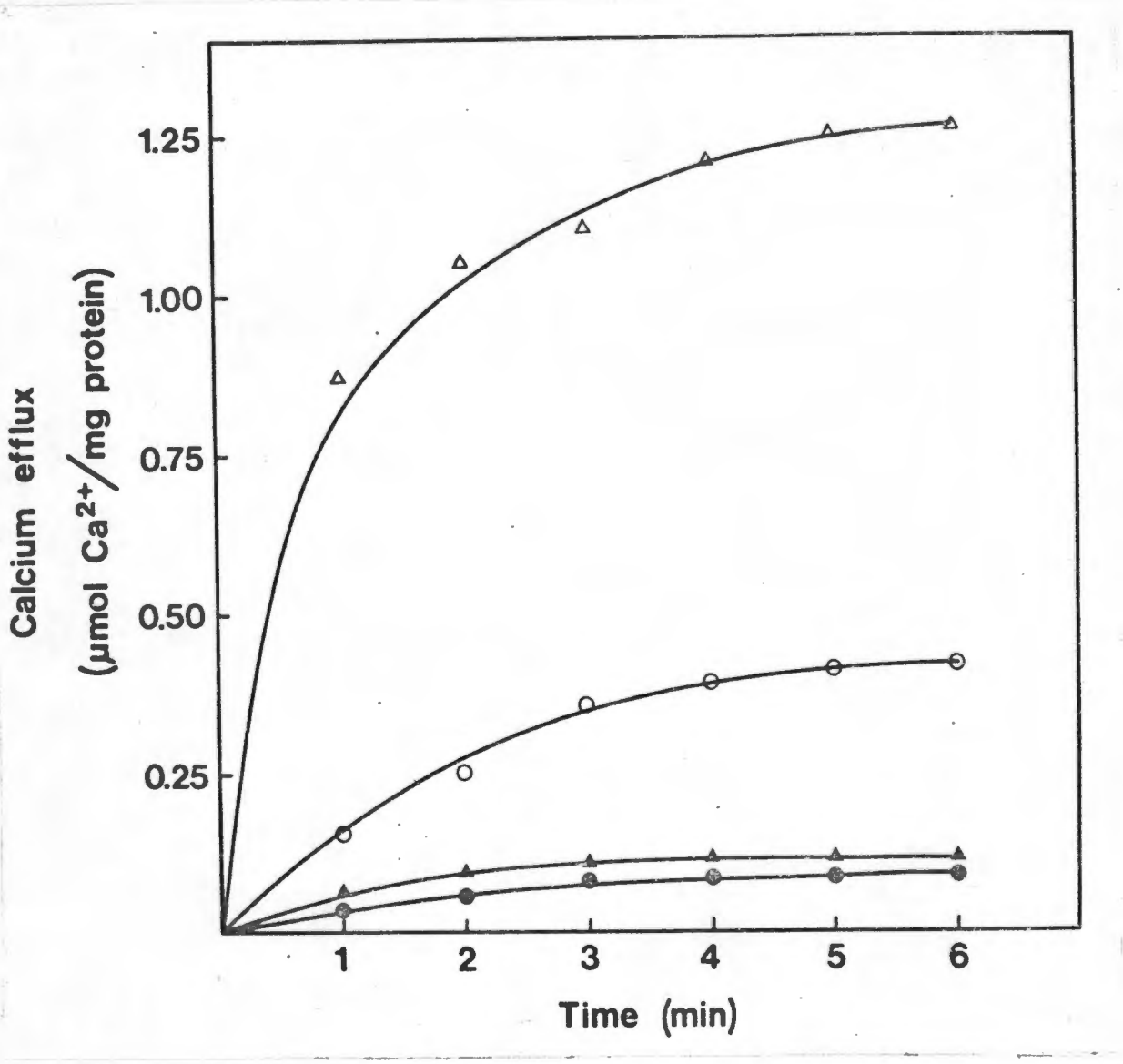

FIGURE 11 EGTA - induced efflux of Ca^{2+} from actively loaded SR vesicles.

SR vesicles were actively loaded for 10 min. in the presence of 50mM potassium phosphate, pH 6,8. Efflux was initiated by the addition of 100mM EGTA to a final concentration of 3,3mM. Zero minutes indicates the addition of EGTA. The temporal efflux of Ca^{2+} was monitored by determining the amount of Ca^{2+} associated with the vesicles by the Millipore filtration method.

KEY

△—△ Control original SR

▲—▲ Acid inactivated control SR

○—○ Reconstituted control SR

●—● Reconstituted acid inactivated SR

TABLE 6. Comparison of initial rates of active Ca^{2+} uptake and EGTA - induced efflux in original, control and reconstituted vesicles.

VESICLE TYPE	Initial rate of uptake (umol/min/mg)	Final amount after 10 min. (umol/mg)	Efflux rate (umol/min/mg)	Final amount after 6 min. (umol/mg)
CONTROL ORIGINAL SR	0,30	2,0	1,6	0,75
CONTROL ACID-INACTIVATED SR	0,04	0,19	0,07	0,08
RECONSTITUTED CONTROL SR	0,15	0,95	0,15	0,53
RECONSTITUTED ACID-INACTIVATED SR	0,08	0,40	0,03	0,30

SR vesicles were actively loaded for 10 min in the presence of 50mM potassium phosphate, 0,12mMKCl, 40mM histidine, pH 6.8, 0,5mM EGTA, 0,5mM $CaCl_2$ and 5mM ATP. Calcium efflux was initiated by the addition of 3,3mM EGTA and the efflux monitored for 6 min (see Materials and Methods).

3.2.5.5. Tightly bound Ca^{2+} in sarcoplasmic reticulum.

The ability of sarcoplasmic reticulum membranes to bind Ca^{2+} tightly was previously shown in this laboratory (Diamond *et al.*, unpublished observations) to be closely associated with Ca^{2+} transport activity in the same membranes. We have used this method to further characterise reconstituted acid inactivated SR vesicles.

Tightly bound Ca^{2+} was assayed in freshly isolated original SR, control and reconstituted vesicles. Figure 12 shows the efflux of Ca^{2+} from vesicles passively loaded with $^{45}CaCl_2$ for 2h at 0°C. Efflux was initiated by diluting SR vesicles 30-fold into an efflux medium containing 1mM EGTA (Materials and Methods). A summary of the results obtained for 2 experiments as calculated from efflux curves similar to those in Figure 12 is presented in Table 7.

Reconstituted control SR bound Ca^{2+} tightly of the same order as that of freshly isolated original SR. Reconstituted SR vesicles bound Ca^{2+} tightly to the same extent although the Ca^{2+} transport activity in the reconstituted acid inactivated SR was approximately 52% that of the reconstituted control SR vesicles. Similarly control original vesicles, although exhibiting double the transport activity of reconstituted vesicles, bound only 50% of the total amount of Ca^{2+} bound by reconstituted vesicle.

It appears from this data that although the ability of SR membranes to bind Ca^{2+} tightly appears to be linked to transport the degree of tight binding is not dependent directly on calcium transport activity.

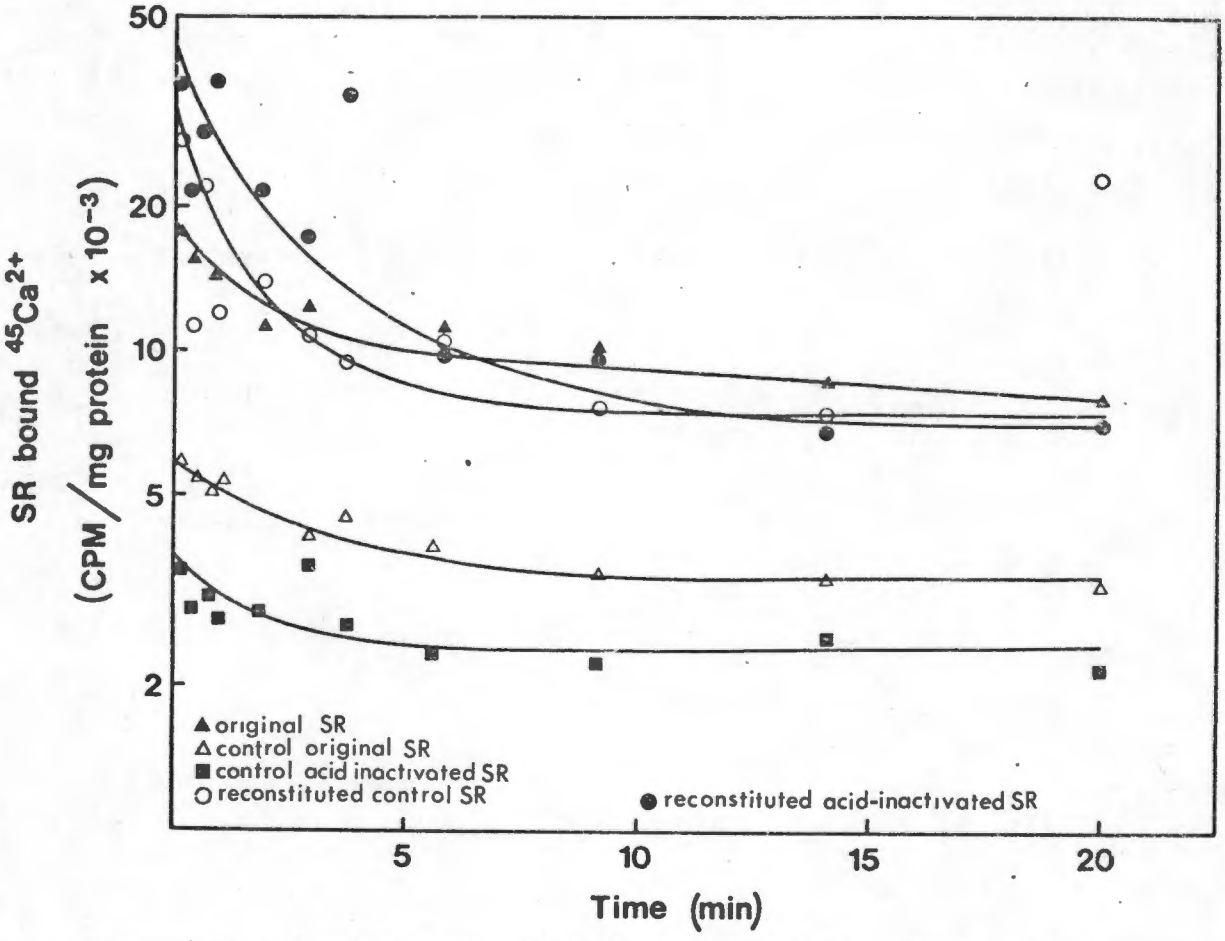

FIGURE 12 Tightly bound calcium in SR membranes.

SR protein was solubilized and reconstituted as described above. After dialysis vesicles were concentrated at 176 000 x g after dialysis and resuspended in 20mM Imidazole pH 7.25, 80mM KCl, 2,5 mM $MgCl_2$.
$^{45}CaCl_2$ was added to a final concentration of 5mM and the vesicles incubated for 2 h at 0°C. Efflux was initiated by diluting SR protein 30-fold into a medium containing 20mM imidazole, 80mMKCl, 1,0mM EGTA. Efflux of Ca^{2+} was monitored by the Millipore filtration method (Materials and Methods).

TABLE 7. Comparison of tightly bound Ca^{2+} in original control and reconstituted vesicles.

VESICLE TYPE		Initial rate of Ca^{2+} uptake (umol/min/mg)	Tightly bound Ca^{2+} (nmol Ca^{2+}/mgprotein)
ORIGINAL SR	1	1,57	2,75
	2	2,12	2,73
RECONSTITUTED CONTROL SR	1	0,15	2,56
	2	0,20	2,37
RECONSTITUTED ACID-INACTIVATED SR	1	0,075	2,39
	2	0,115	2,13
CONTROL ORIGINAL SR	1	0,29	1,23
	2	0,115	2,13
CONTROL ORIGINAL SR	1	0,29	1,23
	2	0,46	1,43
CONTROL ACID-INACTIVATED SR	1	0,04	0,96
	2	0,08	0,82

The results for duplicate experiments are required. DOC-solubilized protein was reconstituted and suspended in 20mM imidazole buffer pH 7.25 containing 80mM KCl and 2,5 mM $MgCl_2$. 100mM $^{45}CaCl_2$ was added to the protein suspension to a final concentration of 5mM. Vesicles were equilibrated for 2h on ice and the tightly bound Ca^{2+} determined as described under 'Materials and Methods'.

3.2.5.6. Tightly bound Adenine Nucleotides

Sarcoplasmic reticulum membranes have been shown to bind adenine nucleotides tightly and it has been suggested (Aderem et al., 1979) that these nucleotides may play a role in transport events of the (Ca^{2+},Mg^{2+}) -ATPase. The ability of reconstituted acid inactivated SR to bind adenine nucleotide tightly has been investigated. Tight nucleotide content is summarised in Table 8. These data show that acid-inactivated SR regain the ability to bind nucleotides tightly following DOC-solubilisation and slow dialysis at room temperature.

TABLE 8. Tightly bound (total) adenine nucleotides in SR vesicles.

VESICLE TYPE	Tightly bound nucleotides (mol/mol ATPase)
ORIGINAL SR	0,25
CONTROL ORIGINAL SR	0,20
ACID-INACTIVATED CONTROL SR	0,07
RECONSTITUTED CONTROL SR	0,34
RECONSTITUTED ACID-INACTIVATED SR	0,24

SR vesicles (1mg/ml) were enriched in nucleotide content by incubation at 30°C for 3 min. in a medium containing 50mM histidine, pH 7.0, 50mMKCl, 5mM $MgCl_2$, 5mM EGTA, 4,7mM $CaCl_2$, 5mM PEP, 30 units/ml pyruvate kinase and 5mM ATP. The reaction was initiated by the addition of SR protein and the reaction terminated by incubation at 0-4°C. Loosely-bound nucleotides were removed from the SR membrane by passage through a Dowex I column (see Materials and Methods).

4.0. DISCUSSION

4.0. D I S C U S S I O N

4.1. Solubilization and reconstitution of isolated sarcoplasmic reticulum:

The numerous descriptions of methods for reconstitution of membrane associated functions, in particularly transport phenomena, indicates that methods of reconstitution are not yet optimal or standardised. In this study calcium transport activity in reconstituted vesicles was lower than that obtained in the original description of Repke and collaborators (1976), on the basis of specific activity measurements. Several factors may have contributed to this discrepancy. For instance the addition or exclusion of sucrose either during solubilization or in the dialysis period has apparently opposing effects. In both this study and that of Repke et al. (1976) the presence of sucrose during solubilization resulted in vesicles exhibiting relatively low transport activities whilst addition of sucrose following solubilization and prior to dialysis resulted in 'tightly coupled' vesicles. Certain anomalies were also noted with the presence or absence of precipitating anions e.g. oxalate or phosphate (Racker, 1972). In this study oxalate did not augment transport activity as originally described by Racker (1972).

However by all criteria, reconstitution of transport activity has been achieved and the characteristics of reconstituted SR vesicles are similar to those described in the literature. Reconstituted preparations would appear therefore to be valid models for further study. It is envisaged that some modification of deoxycholate concentrations, sucrose and rates of dialysis could lead to more active preparations. In this respect it is noteworthy that considerable amounts of deoxycholate remain associated with reconstituted material following prolonged dialysis (Fig 4). This result has not previously been reported. Residual deoxycholate, closely associated with SR membranes would be expected to have a deleterious effect on transport activity. In this study only a

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restricted range of deoxycholate concentrations were tested for optimal conditions of solubilization. A more extensive investigation should indicate optimal concentrations of deoxycholate for solubilization without adversely affecting ATPase activity or transport activity in reconstituted material.

Electron-micrographs of reconstituted vesicles (Fig 5) show sealed vesicles which appear to be somewhat larger than original vesicles. This has previously been commented upon (Repke et al., 1976) and is indicated functionally by increased capacity of vesicles actively loaded in the presence of ATP.

An interesting facet of reconstituted studies concerns the protein profile of reconstituted vesicles as compared with original vesicles. SDS polyacrylamide gel electrophoresis of reconstituted vesicles shows that there is a relative lack of calsequestrin but that the M55 protein becomes incorporated during reconstitution. Similar findings were noted by Repke et al. (1976). It is generally assumed (See Section 1.2.) that both the M55 and calsequestrin are situated on the inside of the vesicles and there is thus no reason to presuppose that there is differential solubilization during reconstitution procedure. Possibly there is some association between the ATPase and M55 proteins which persists when the membrane is solubilized in the presence of deoxycholate and that there is weaker or no association between the ATPase and calsequestrin.

4.2. Reconstitution of acid-inactivated SR.

It has previously been shown in this laboratory (Berman et al., 1977) that acid-inactivation of calcium transport activity is irreversible and cannot be reversed by prolonged incubation at 0°C or at room temperature. This study, as originally conceived, was designed to investigate the possibility that the irreversibility of acid-inactivation was due to constraints imposed upon the ATPase polypeptide by phospholipid molecules in the membrane in close apposition to the hydrophobic surface of the transmembrane protein. This study has in fact shown that significant transport

activity/...

activity can be regained by acid-inactivated SR vesicles following reconstitution by DOC-solubilisation and subsequent slow dialysis.

Electrophoretic analysis of reconstituted control and reconstituted acid-inactivated vesicles show no obvious differences suggesting that reconstitution has been identical for both species. In particular, the reconstituted vesicles showed an apparent enhancement of ATPase content of the order of 20% as determined by protein extraction data and densitometric traces of SDS-gels (Table 2).

One possible explanation for the reconstitution of calcium transport activity in solubilized acid-inactivated SR is that acid-inactivation results in an increased leakiness of the SR membrane to Ca^{2+} and this leakiness is repaired in the reconstitution process. Thus the apparent uncoupling of calcium transport activity induced by mild acid exposure can be reversed by reconstitution. Previous studies however, also from this laboratory (Mc Intosch and Berman, 1978) have presented strong evidence that the apparent uncoupling by acid is not due to increased permeability of the membrane to Ca^{2+}. The major evidence for this is that Ca^{2+} binding to high affinity sites identical with those on the exterior surface of the ATPase polypeptide chain and which activate transport specifically protect against acid-inactivation, i.e. protection is mediated via the ATPase protein itself and not via Ca^{2+} binding to non-specific sites on the polar headgroups of phospholipids of the lipid portion of the membrane through which Ca^{2+} ions might passively permeate.

Another possible explanation of the data is that reconstitution procedure selects out a specific population of original vesicles which have not been inactivated. In other words, in a typical experiment where for example 10% of the residual transport activity remain, SR vesicles isolated as reconstituted vesicles may represent this residual 10% active fraction. This possibility can be excluded by the fact that following DOC-treatment all material sedimenting within 1h at 176 000 x g was discarded following solubilization, and only the soluble

supernatant/...

supernatant containing solubilised protein and phospholipids was carried through the dialysis procedure. Reconstituted vesicles could only have arisen through the reformation of membranes with associated protein from components in the soluble supernatant and subsequent sealing off of intact vesicles. It is concluded therefore that there was an equal likelihood of inactivated and active protein being associated with lipid in the reconstituted vesicles. If there were preferential incorporation of active protein into reconstituted vesicles (i.e. active with respect to intramolecular coupling and transport) , one would have expected the recovery of reconstituted vesicles to be 10 times greater for reconstituted control SR as for reconstituted acid-inactivated SR in which there was only 10% of residual transport. In fact the recovery of protein in reconstituted control and reconstituted acid-inactivated preparations was identical indicating no preferential incorporation of uninactivated vs inactivated ATPase into reconstituted vesicles.

Both Ca-dependent ATPase and ATP-dependent calcium transport activities were decreased in reconstituted control and reconstituted acid-inactivated vesicles. In contrast control - original and control acid-inactivated vesicles showed elevated Ca^{2+}-dependent ATPase activities. However, the additive effect of low ATPase and low calcium transport activities in reconstituted vesicles resulted in relatively good Ca:ATPase coupling ratios in both the reconstituted vesicles. Control-original vesicles, while exhibiting relatively high calcium transport activities exhibited even higher ATPase activities leading to low Ca:ATPase coupling ratios (Table 3,4). The Ca:ATPase coupling ratios as obtained for reconstituted vesicles is in agreement with results of Repke *et al.* (1976).

Solubilization of SR protein and subsequent slow dialysis at room temperature leads to decreased Mg^{2+}-dependent and Ca^{2+}-dependent ATPase activities, although the Ca^{2+}-dependent ATPase activity becomes more depressed (Table 5) suggesting that the Ca -dependent ATPase activity is more affected

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by solubilising concentrations of deoxycholate. Neutralisation of the calcium gradient by incorporation of calcium specific ionophores into SR membrane results in ionophoric stimulation of the same order as observed in native membranes (Table 5). This suggests that the low Ca^{2+} -dependent ATPase activity observed in reconstituted vesicles (Table 4) is not a function of a tight coupling of calcium transport to ATPase activity but rather an inactivation of the (Ca^{2+},Mg^{2+}) ATPase due to prolonged dialysis at solubilisation concentrations of deoxycholate.

EGTA-induced efflux of Ca^{2+} from SR vesicles actively loaded in the presence of ATP and 50mM potassium phosphate showed slower rates of efflux in both reconstituted-control and reconstituted acid-inactivated vesicles, (Fig 11). The lower rates of Ca^{2+} -induced efflux as well as the relatively high Ca:ATPase coupling ratios in reconstituted vesicles could reflect a low Ca^{2+} permeability of the reconstituted sarcoplasmic reticulum. These results are in agreement with those of Repke et al. (1976).

The ability of sarcoplasmic reticular membranes to bind Calcium tightly was previously shown to be closely related to Ca^{2+} transport in the same membranes (Diamond et al, unpublished observations). Original SR and acid-inactivated SR subjected to deoxycholate solubilisation and slow dialysis at room temperature regain their ability to bind Ca^{2+} tightly (Fig 12, Table 7). Acid-inactivated sarcoplasmic reticulum was shown to lose the ability to bind Ca^{2+} tightly.

Similarly, acid-inactivated SR membranes have been shown to regain their ability to bind adenine nucleotides tightly (Table 8) following solubilization and reconstitution. It has been shown (Aderem et al, 1979) that the ability of SR membranes to bind nucleotides tightly is correlated with the ability of the same membranes to transport Ca^{2+} suggesting that these nucleotides play a role in Ca^{2+} transport.

Acid-inactivation appears to change the conformation of the (Ca^{2+},Mg^{2+}) ATPase leading to an inability of SR membranes

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to transport calcium and a related inability to bind Ca^{2+} or adenine nucleotides tightly. The loss of these functions following acid-treatment could be a manifestation of a change in the micro-environment of the (Ca^{2+},Mg^{2+}) -ATPase, more specifically a change in the lipid-protein interactions around the ATPase. From these studies it would appear that DOC-solubilisation removes the restraints placed on the inactivated protein and allow it to reassume its native conformation.

5.0. BIBLIOGRAPHY

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