Physiological Reviews

Published and Copyright by
The American Physiological Society *Vol. 58, No. 1, January 1978*

Molecular Mechanism of Active Calcium Transport by Sarcoplasmic Reticulum

MICHIHIKO TADA, TAIBO YAMAMOTO, AND YUJI TONOMURA

Department of Biology, Faculty of Science, Osaka University, Toyonaka, and First Department of Medicine, Osaka University School of Medicine, Fukushima, Osaka, Japan

I. INTRODUCTION

Active transport of calcium ions across the membranes of sarcoplasmic reticulum has received much attention for the past several years from biophysical and biochemical points of view, since this membrane system provides one of the simplest and most effective ways to gain insight into the molecular mode of energy transduction. In this review we give our overview of the recently evolving molecular concepts obtained from studies of this energy-transducing system and propose a molecular model to explain the mechanism of transduction of chemical energy into osmotic work. Excellent reviews of early work on the calcium transport of this membrane system are provided by Hasselbach (106), A. Weber (392), and Ebashi et al. (65, 66). Reviews by Inesi (136), Martonosi (228, 229), Tonomura (364), Hasselbach (107, 108), and MacLennan and Holland (201, 202) give details of more recent developments.

A. Historical Overview

A factor capable of inducing relaxation of myofibrillar bundles was first found in an aqueous extract of muscle by Marsh (220, 221), whose observations were subsequently confirmed by Bendall (15, 16) and Hasselbach and H. H. Weber (115). Kumagai et al. (180) and Ebashi (60) showed that the relaxing activity was associated with the Kielley-Meyerhof particulate fraction of muscle, which possessed a Mg^{2+}-dependent ATPase activity (167, 168). The microsomal fraction that possessed this relaxing activity consisted of membrane vesicles (67, 259–261), which presumably were derived from the sarcoplasmic reticulum, a membrane-limited reticular structure within muscle cells (see below). Hasselbach and Makinose (111) and Ebashi and Lipman (62, 67) demonstrated that the microsomal fraction was capable of removing a significant amount of Ca^{2+} from the medium in the presence of ATP and Mg^{2+}. Hasselbach and Makinose (111, 113) demonstrated an ATPase activity in the microsomal vesicles that was activated by the simultaneous additions of Ca^{2+} and Mg^{2+}. This fraction was also found to catalyze an ATP-ADP exchange reaction in the presence of Ca^{2+} and Mg^{2+} (67, 112, 113).

Detailed studies by Porter and co-workers (17, 83, 289, 290) and Peachey (286, 287) demonstrated that the sarcoplasmic reticulum consists of a membrane-limited reticular structure of continuous vesicles, tubules, and cisternae that forms a network surrounding the myofibrils. They also found another tubular structure running perpendicularly to the long axis of muscle (the *transverse tubule* or *T system*), which forms a junction with the terminal cisterna, a part of the sarcoplasmic reticulum that is thickened to form a continuous sac. The membranes of the T system were found to be continuous with the surface membrane (*sarcolemma*), indicating that its lumen is a continuation of the extracellular space (69, 83, 125). Porter (289) and Muscatello et al. (259, 260) were among the first to suggest that microsomal vesicles possessing relaxing activity are derived from the sarcoplasmic reticulum.

That the injection of Ca^{2+} into myoplasm can produce local contraction was first observed by Kamada and Kinoshita (153) and Heilbrunn and Wiercinski (116). The latter authors suggested that Ca^{2+} ions are the physiological inducer of the contractile process. The role of Ca^{2+} in inducing muscle contraction was not clarified until a number of investigators proved that a minute amount of Ca^{2+} exerts critical control over the contractility of glycerinated muscle fiber (21, 22, 388, 389), the ATPase activities of isolated myofibrils (396) and natural actomyosin (390, 391, 395, 401), contraction of myofibrils (395, 396), and superprecipitation of actomyosin (61, 64, 401).

These observations that muscle cells possess both a calcium-sensitive contractile system and a calcium-accumulating membrane system have led to the physiological concept that Ca^{2+} released from the sarcoplasmic reticulum can induce muscle contraction and Ca^{2+} accumulation by the sarcoplasmic reticulum can cause muscle to relax. This concept is substantiated further by the findings described below.

A. F. Huxley and Taylor (124) observed that a weak electrical current applied to the surface of a muscle fiber can produce localized contraction when applied selectively to the region corresponding to the opening of the T system. They suggested that the T system may mediate the transmission of the action potential to the interior of the fiber, thus causing localized activation of the sarcoplasmic reticulum. Their contention found support when selective damage to the T system by hypertonic glycerol was found to cause irreversible loss of the twitch response (90, 122). A rapid increase in intracellular Ca^{2+} concentration immediately after electrical stimulation of muscle fiber was demonstrated by Jöbsis and O'Conner (149), who injected intracellularly the metallochromic indicator murexide, and by Ashley and Ridgway (6–8, 308), who injected the bioluminescent protein Aequorin, to monitor intracellular Ca^{2+} concentrations. Ebashi and co-workers demonstrated the existence of troponin, a calcium-receptive protein, in the thin filaments of the myofibrils (65, 66). Recent reviews discuss the subsequent development of the molecular concept that the interactions between actin, myosin, and ATP are regulated by Ca^{2+} through its interactions with the calcium-receptive troponin-tropomyosin system (63, 363, 400).

It appears likely that release and subsequent reaccumulation of Ca^{2+} by the sarcoplasmic reticulum take place in each cycle of contraction and relaxation. Indeed, Endo and co-workers (71, 72) demonstrated that depolarization of the sarcoplasmic reticulum in Natori's skinned-fiber preparation (269) caused calcium release that initiated contraction. Recent developments in studies of calcium release from the sarcoplasmic reticulum have been extensively reviewed by Endo (70). The ATP-supported uptake of Ca^{2+} by microsomal vesicles, which is considered to represent the in vitro manifestation of the calcium accumulation by the sarcoplasmic reticulum, has been proven by a number of investigators to represent the calcium transport across the membranes of sarcoplasmic reticulum against a concentration gradient (65, 66, 106, 392).

B. Bioenergetically Favorable Characteristics of Calcium Transport

The isolated membranes of the sarcoplasmic reticulum have proven suitable for the study of the molecular mechanisms of active cation transport since this system possesses several distinct bioenergetic features lacking in other energy-transducing systems. These characteristics include:

1) A high content of the ATPase protein; it makes up more than two-thirds of the total protein.

2) Tightly coupled ATP hydrolysis and calcium transport in which 2 mol of calcium are transported for each mole of ATP hydrolyzed under a variety of conditions.

3) A clear distinction between the inside and outside of the membrane so that Ca^{2+}, which stimulates ATP hydrolysis only from outside the membrane, is translocated to the inside. In contrast, agents like ATP, ADP,

P_i, ethylenediaminetetraacetic acid (EDTA), and ethylene glycol bis-(β-aminoethylether)*N*,*N*′-tetraacetic acid (EGTA) react only from the outside and are completely excluded from the vesicular lumen.

4) Easy control of the Ca^{2+} concentration in the medium by the use of the chelating agent EGTA, and of that within the interior of the membrane vesicle by the use of membrane-permeable calcium-precipitating anions like oxalate, thus making the kinetic analysis of calcium transport much simpler than that of other membrane systems like sodium and potassium transport and mitochondrial and chloroplast proton transport.

5) Ability of certain detergents to make the membrane leaky, without major effects on enzyme activity.

6) A readily measured trichloroacetic acid (TCA)-stable phosphoprotein reaction intermediate that facilitates the characterization of elementary steps of the reaction.

7) Complete reversibility of the active transport process.

These favorable features of this system have led to the rapid and detailed evolution of molecular concepts in the bioenergetics of cation transport. Because of these recent developments, we are now able to argue realistically about the molecular mechanism of chemiosmotic coupling.

C. Preview

A brief preview of this article is now presented to orient the reader in following the trail we provide that leads to a molecular concept of cation transport.

In the first few sections our main concern is to summarize the current knowledge of the major properties of the ATPase protein of the sarcoplasmic reticulum. These include its structural features within the membrane (sect. II), its purification, and the reconstitution of its physiological function (sect. III). The latter achievement, in which the calcium pump was reconstituted from the purified ATPase and phospholipids, is one of the most remarkable successes in bioenergetics in recent years. In preparation for our discussion of the role of the ATPase as an energy transducer in the calcium pump, section IV outlines the characteristics of previously proposed models for cation transport. Calcium binding by the sarcoplasmic reticulum and by its individual components and the distribution of Ca^{2+} within these membrane vesicles are described in section V, emphasis being placed on the fact that the ATPase protein binds Ca^{2+} with high affinity. Section VI summarizes the steady-state properties of Ca^{2+}-Mg^{2+}-dependent ATP hydrolysis by the sarcoplasmic reticulum and the purified ATPase protein. In sections VII and VIII, we are largely concerned with the most important properties of the ATPase in connection with three elementary steps in the active transport of Ca^{2+}, i.e., recognition, translocation, and release of Ca^{2+}. These two sections, which document the reaction mechanism of the ATPase and its relation to the calcium transport, should reveal the elementary steps of the ATPase reaction and the change in affinities of the ATPase protein for cations (sect.

VII) and the coupling of the formation of the reaction intermediate with calcium translocation (sect. VIII). Section IX describes several modes of regulation that could control the elementary processes of the ATPase reaction, as well as a new type of regulation of calcium transport in cardiac sarcoplasmic reticulum that is mediated by the cyclic AMP-dependent protein kinase system. Section X documents energetics of the reversibility of the calcium pump, another successful achievement in the bioenergetics of cation transport. As a final step in attempting to devise a molecular model of cation transport, section XI details dynamic properties of the ATPase protein, lipid, and their interactions as they are manifested in the calcium-pumping function of the membrane. We also document our current knowledge on the submolecular structure of the ATPase that should enable us to define more precisely the molecular mechanism of calcium transport. Based on a large body of accumulated knowledge, section XII discusses the two major types of molecular model for cation transport: the rotatory-carrier model and the mobile-pore model, as they may explain the molecular mechanism for energy transduction in calcium transport by the sarcoplasmic reticulum. In concluding this article (sect. XIII) we describe the similarities between the basic mechanisms of the molecular operation in two major energy-transducing systems: cation transport and muscle contraction.

As will be clear from this preview, this article is not intended to give an overall view of the physiology and biochemistry of cation transport by the sarcoplasmic reticulum, but instead is intended to summarize problems concerning the molecular bioenergetics of active Ca^{2+} transport across this membrane. It is hoped that this review will stimulate the reader and contribute to a better understanding of the fundamental processes in the molecular mechanism of energy transduction.

II. ULTRASTRUCTURE OF ISOLATED SARCOPLASMIC RETICULUM

The membranes of sarcoplasmic reticulum are composed of several proteins and lipids as revealed by gel electrophoresis and gel filtration. Structural studies with electron microscopy, X-ray diffraction, and spin-labeling techniques demonstrate that they are organized in a manner essentially compatible with the fluid mosaic model of Singer and Nicolson (331). These ultrastructural features of sarcoplasmic reticulum membranes are summarized in this section, with the main emphasis on the documentation of the molecular structure of the ATPase, the major protein component of this membrane.

A. Isolation of Sarcoplasmic Reticulum

The sarcoplasmic reticulum vesicles were isolated in microsomal fractions by differential centrifugation of a muscle homogenate in isotonic salt solutions (291). Contaminating actomyosin was removed by extracting this

preparation with 0.6 M KCl, which dissolves actomyosin without dissolution of sarcoplasmic reticulum proteins (225). These procedures, which represent the standard method employed by many laboratories, are occasionally modified (130, 244) by inclusion of sucrose in the extraction medium. The membranes thus prepared are relatively pure and are considered to retain most of the physiological characteristics of the sarcoplasmic reticulum in situ. Recently, Meissner (246) reported that the sarcoplasmic reticulum isolated by the standard procedures consisted of a heterogenous population of vesicles, which could be separated into light and heavy populations on linear sucrose gradient by centrifugation at 28,000 rpm for 20 h. The differences in buoyant densities appeared to reflect differences in phospholipid-to-protein ratios. Electron-microscope and calcium-binding studies suggested that these fractions were derived from different parts of the sarcoplasmic reticulum, such as the longitudinal sections and terminal cisternae (246). These observations support a previous suggestion by Winegrad (403a), who contended that the longitudinal sections and terminal cisternae of sarcoplasmic reticulum may function as sites for accumulation of Ca^{2+} and for release of Ca^{2+}, respectively. The presence of the calcium-binding proteins in terminal cisternae (heavy vesicles) (251) would justify the presence of a sink for the Ca^{2+} in that region of sarcoplasmic reticulum. Since this is beyond the scope of the present article, the reader is referred to an excellent review by Endo (70).

B. Lipid Composition

Phospholipids make up about 80% of the total lipid on a molar basis; the remainder is neutral lipid, of which 95% is cholesterol. As summarized in Table 1, 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%). Although phosphatidylcholine alone was sufficient to activate the ATPase activity, and phosphatidylethanolamine was also required for the maximal activity of calcium transport (175; see sect. III*B*), it is not exactly understood how the diversity of phospholipids found in the native membrane are required for the transport function. Of about 90 lipid molecules associated with each molecule of ATPase in native sarcoplasmic reticulum, about 30 molecules were found to interact directly with this protein (117, 384–387). The rest of the lipid molecules were considered to contribute to the fluid bilayer characteristics. Further discussion of the role of lipid in the ATPase and calcium transport activities is found in sections III*B* and XI*A*.

C. Protein Composition

The protein composition of sarcoplasmic reticulum has been extensively

TABLE 1. *Phospholipid composition of sarcoplasmic reticulum and purified ATPase preparation*

	Martonosi et al. (233)	Meissner and Fleischer (248)	MacLennan et al. (205)	Owens et al. (277)	Sanslone et al. (313)	Waku et al. (382)
Phosphatidylcholine	65.0	72.7	64.9 (65.9)	67.0 (63.9)	69.6	70.8
Phosphatidylethanolamine	12.3	13.5	18.8 (17.1)	17.6 (20.4)	18.4	16.5
Phosphatidylinositol		8.7		9.2 (9.4)	9.3	2.3
Phosphatidylserine	6.2	1.8	11.4 (11.9)	2.1 (2.9)		1.7
Sphingomyelin		1.0	4.6 (4.7)	3.9 (3.1)		6.2
Cardiolipin		0.3	0.3 (0.4)	0.1 (0.0)		0.3
Phosphatidic acid		0.2				trace
Unidentified phospholipid	16.5	1.8			2.7	3.0
Total	100.0	100.0	100.0 (100.0)	99.9 (99.7)	100.0	100.8

Values represent percent of total phospholipids. Numbers in parentheses in reports by MacLennan et al. (205) and Owens et al. (277) give phospholipid composition of purified ATPase preparation.

investigated and four major proteins have been identified (130, 136, 204, 207, 226, 238, 247). All were considered to be related to some extent to the calcium transport activity. The ATPase protein with a molecular weight of about 100,000 daltons accounts for up to 90% of the total protein (246). Other proteins are calsequestrin, high-affinity calcium-binding protein, and proteolipid. Structural properties of these proteins are documented below (sect. II, *D–F*). Sarcoplasmic reticulum membranes also contain additional minor proteins whose properties are not well defined (200, 207). The protein composition of sarcoplasmic reticulum membranes, prepared from rabbit fast-contracting skeletal muscle by the usual method of differential centrifugation, was estimated by measuring the density profile of stained proteins after disk-gel electrophoresis. Many reports indicate that the ATPase accounted for 60–80% of the total protein (39, 130, 136, 238, 247, 406), calsequestrin accounted for 5–19% (136, 247), and the high-affinity calcium-binding protein for 5–12% (136, 247). However, Meissner (246) found that sarcoplasmic reticulum of high and low density, separated on a sucrose-density gradient, had different protein compositions. He reported that light vesicles were composed largely of ATPase (about 90%) whereas heavy vesicles contained the ATPase (55–60%), calsequestrin (20–25%), and the high-affinity calcium-binding protein (5–7%).

D. Molecular Structure and Distribution of Proteins Within the Membrane

In documenting the molecular structure and distribution of the proteins

within the membranes of sarcoplasmic reticulum, our main interests focus on the structural features of the ATPase, the major protein of this membrane, which has been identified as a single polypeptide of about 100,000 daltons (sect. III*A*). This protein appears to play a significant role in the energy transduction of calcium transport and has been proposed to serve as the carrier in the translocation of Ca^{2+} (see sect. IV). These hypotheses require that two important questions be answered: whether this molecule is exposed to both inner and outer surfaces of the membrane, and whether this molecule exists as a monomer or an oligomer. The Na^+-K^+-dependent ATPase has been shown to span the plasma membrane, exposing its strophanthidin-binding site to the outer surface (31, 312) and its antigen-binding (183) and ATPase sites (320, 402) to the inner surface. In the Ca^{2+}-dependent ATPase of the sarcoplasmic reticulum, Stewart et al. (338) showed that part of this polypeptide was exposed to the outer surface of the membrane by demonstrating the binding of antibodies against the ATPase and its proteolytic fragments to the membrane of sarcoplasmic reticulum (see sect. XI*B*). A similar conclusion was reached earlier by Hasselbach and Elfvin (109), who examined electron microscopically the binding of Hg-phenylazoferritin to the membrane of sarcoplasmic reticulum. However, it was not clear whether part of this protein was also exposed to the internal surface of the membrane.

The molecular structure and distribution of the ATPase and other proteins within the membranes of sarcoplasmic reticulum were assessed to some extent by the use of the structural probes. X-ray diffraction studies of sarcoplasmic reticulum vesicles, packed by centrifugation, indicate that proteins were asymmetrically distributed across the membrane (57, 58, 194, 404). However, two groups of investigators have reached different conclusions in that Worthington and Liu (194, 404) found a denser distribution of proteins on the luminal surface, whereas Dupont and co-workers (57, 58) found these proteins to be concentrated on the external surface. This discrepancy may reflect the lack of critical means by which the internal and external phases of the bilayer membranes were determined. Freeze fracture of sarcoplasmic reticulum was first used by Deamer and Baskin (40), who found that particles 8–9 nm in diameter were embedded within the membrane, the majority being contained in the cytoplasmic leaflet. They proposed, without direct evidence, that each particle represented the structural feature of the transport ATPase. MacLennan et al. (205) and Deamer (39) reported that 9-nm particles were found in the fracture faces of the membranous structure formed by the purified ATPase, although they were found to be present symmetrically in the two fracture faces (205, 278) in contrast to the intact sarcoplasmic reticulum. Whether each of these particles represents a monomer or an oligomer of the ATPase molecule (218, 314, 315) is still controversial, but it is quite likely that the particles represent distinct intramembranous structures rather than artifacts. A number of quantitative determinations of particle distribution on the fracture faces demonstrated that the cytoplasmic leaflet contained the majority of the particles (278, 315, 336, 361), a finding that is consistent with X-ray data that indicate an asymmetry of protein distribution across the membrane.

Electron-microscopic observation of negatively stained membranes of sarcoplasmic reticulum permit another view of the external surface of the membrane, where at least part of the ATPase molecule can be assumed to be exposed. Ikemoto et al. (134) and Inesi and Asai (138) found that the surface of the sarcoplasmic reticulum membranes was covered with particles about 4 nm in diameter, which were later observed to be connected with stalks about 2 nm in diameter (133). Trypsin digestion of the membrane abolished the surface particles, indicating that the headpieces were protein in nature. Ikemoto et al. (134) reported that the surface particles were removed without accompanying loss of the ATPase, and calcium transport activities when 1 M sucrose was present in the digestion medium, suggesting that the surface particles are not related to the ATPase (133). However, these intriguing effects of trypsin on the surface particles and the ATPase activity were later reexamined more extensively. In contrast to the earlier conclusion, these observations indicated that trypsin digestion, either short term or in the presence of sucrose or ATP, abolished neither surface particles nor the ATPase activity (253, 336, 337, 359), and that the surface particles and the ATPase activity were both lost only when the membrane was extensively digested by trypsin (253, 336, 337). These observations support the view that surface particles represent at least part of the ATPase molecule.

These studies on the ultrastructural characteristics of the sarcoplasmic reticulum membranes suggest that the main body of the globular structure, which presumably represents part of the ATPase, is localized largely in the cytoplasmic leaflet of the bilayer membrane and that part of this structure is also exposed to the external surface of the membrane. However, there is not enough evidence to support the view that the ATPase molecule spans the membrane and that a portion is exposed to the internal surface.

In order to understand the functional role of the ATPase, it is also important to determine whether the individual ATPase molecule functions as a monomer or whether it has to form an oligomer to exert its physiological function. Scales and Inesi (315) found a discrepancy between the densities of the surface particles and the 9-nm intramembranous particles and suggested that this discrepancy was probably due partly to the formation of oligomers containing three or four hydrophobic ends that appeared as single 9-nm particles. However, this discrepancy can also be explained by variable penetration into the membrane of the polypeptide chain of the ATPase. Le Maire et al. (192) showed that fully active Ca^{2+}-dependent ATPase of sarcoplasmic reticulum solubilized in the nonionic detergent Tween 80 (monooleate), has a minimal molecular weight of about 400,000, corresponding to a trimer or tetramer of the ATPase. However, it is premature to conclude from these experiments on the solubilized ATPase that the polypeptide chain of the ATPase within the native membrane is an oligomer. This problem is reexamined later in this review (sect. XI*D* and XII).

E. Proteolipid

MacLennan et al. (207) reported that proteolipid represents another

constituent of the intrinsic proteins of the sarcoplasmic reticulum. This component was identified as a band with a molecular weight of about 6000, which was faintly stained by Coomassie brilliant blue after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The purified ATPase preparation contained this component to some extent. Amino acid analysis of the proteolipid purified by the procedures of Folch and Lees (82) indicated that, unless this proteolipid is heterogeneous, the molecular weight of 6000 determined by gel electrophoresis was inaccurate since half-residues of lysine and histidine were obtained if a molecular weight less than 12,000 was assumed (207). If a minimal molecular weight of 12,000 was assumed, then there were 1–2 mol of fatty acid apparently covalently bonded per mole of protein. Laggner and co-workers (185–187) suggested that proteolipid existed in the central region of the lipid bilayer, forming a framework that reduced the fluidity of the hydrophobic region of the bilayer. Racker and Eytan (306) demonstrated that the inclusion of proteolipid within the membranes reconstituted from the purified ATPase and phospholipids markedly improved the efficiency of calcium transport, i.e., the ratio of Ca^{2+} transported per ATP hydrolyzed. They suggested that proteolipid served as a coupling factor. However, the physiological role of this component is yet to be clarified.

F. Calsequestrin and High-Affinity Calcium-Binding Protein

In contrast to the ATPase and proteolipid, which are intrinsic to the membrane structure, other proteins of sarcoplasmic reticulum, such as calsequestrin and high-affinity calcium-binding protein, are bound much more loosely to the membrane and are easily extracted by mild treatment with detergents (130–132, 206, 207, 238, 247, 248, 274). These proteins thus may be referred to as peripheral or extrinsic protein (331).

Calsequestrin is a protein of strong negative charge, with a molecular weight originally reported to be 44,000 (206). Later reports on its molecular weight were not consistent, and values varied between 46,500 and 65,000 daltons (130, 131, 199, 247, 275). The apparent molecular weight of calsequestrin was found to change after minor changes in the conditions during electrophoresis (247). It is acidic in amino acid composition, being suitable for sequestering Ca^{2+} (see sect. v*C*). Calsequestrin is readily soluble in water, but on addition of Ca^{2+} forms an insoluble precipitate. Studies with antibody binding indicated that calsequestrin is localized within the vesicular lumen (338), although an outer localization is also suggested by indirect evidence (359). Since calsequestrin was not essential in reconstituting the active calcium transport (sect. III*B*), it was considered to act as a calcium-storage site within the vesicle, at which Ca^{2+} translocated from outside the vesicle could bind, rather than participating as an integral part of the calcium pump. However, Repke et al. (307), who reconstituted an active calcium pump of sarcoplasmic reticulum, presented rather strong evidence against a role for calsequestrin in calcium storage.

The other protein in this category has a molecular weight of 55,000 and was referred to as high-affinity calcium-binding protein (274) or M_{55} protein (247). This protein was detached from the membrane by extraction with deoxycholate. Although antibody binding studies indicated its internal localization (338), its functional role is yet to be clarified.

Finally, the protein and lipid composition of sarcoplasmic reticulum has been found to vary during the differentiation of skeletal muscle cells (19, 121, 232, 420). Studies on the biosynthesis of the sarcoplasmic reticulum thus will provide useful information on the ultrastructural characteristics (146, 361). Since this is beyond the scope of the present article, the reader is referred to an excellent review by Martonosi (231).

III. RECONSTITUTION OF ACTIVE CALCIUM TRANSPORT

Since various kinds of lipid and proteins are found in the membranes of sarcoplasmic reticulum (sect. II*B*), it is of interest to define which component(s) is essential in maintaining the calcium transport of the membrane. In this section, therefore, we give details of recent studies on the reconstitution of sarcoplasmic reticulum vesicles from purified components, prior to the documentation in subsequent sections on the molecular mechanism of calcium transport. As was stressed previously (sect. I*B*), the relative simplicity of the composition of this membrane system has made possible rapid progress in the successful reconstitution of functioning membranes.

A. Purification of ATPase Enzyme

The purification of the major protein component, the ATPase, without loss of activity was successfully achieved by MacLennan (198), who treated the sarcoplasmic reticulum membrane with deoxycholate (0.1 mg/mg protein) in the presence of 1 M KCl. This step eliminated most extrinsic and contaminating proteins. The ATPase protein, remaining in the membrane, was subsequently dissolved with higher concentration of deoxycholate (0.5 mg/mg protein) and fractionated with ammonium acetate. Similar attempts to purify the ATPase protein were made by Ikemoto, Meissner et al., Deamer, Warren et al., and le Maire et al., who solubilized the membrane with detergents like Triton X-100 (130), deoxycholate (248, 385), lysolecithin (39), or dodecyloctaoxy ethylene glycol monoether (192). They eliminated other proteins either by precipitation with Ca^{2+} (130), by column chromatography on Sepharose 4B (130, 192), or by centrifugation on sucrose density gradients (39, 248, 385). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of these preparations revealed that they consisted mainly of a protein of molecular weight about 100,000. Lipid and a proteolipid of low molecular weight were associated with the purified ATPase (204, 306). Amino acid analysis of the purified ATPase (205, 238, 247, 360) indicated that polar amino acids accounted for a rather large fraction of the total amino acids. Thus, glutamic and aspartic acids accounted for about 24% of the total amino acids, outnumbering other polar amino acids, lysine and

arginine, which accounted for another 10% of the total. Cysteine and histidine were present in relatively lower amounts, 24 and 8 mol/mol of enzyme (about 3 and 1%), respectively. The purified ATPase prepared by MacLennan's procedures (198) contained about 530 μg phospholipid/mg protein, an amount almost equal to that found in the intact sarcoplasmic reticulum (205). The phospholipid composition of the purified ATPase was also identical to that of the intact membranes (see Table 1).

The purified ATPase protein solubilized in the presence of detergents formed vesicles on removal of these solubilizing agents by gel filtration or by simple dilution with aqueous solution (319). Electron-microscopic observations revealed that the reformed membrane exhibited structures similar to the intact membrane with a few exceptional features (see sect. II*D*). The purified ATPase catalyzed a Ca^{2+}-Mg^{2+}-dependent ATP hydrolysis (198, 207) and ATP-ADP exchange reaction (198). The purified enzyme also formed a phosphorylated intermediate on the addition of ATP (see sect. VI*E* and VII). In the absence of ATP, this preparation was capable of binding Ca^{2+} almost equivalent to that found in the intact sarcoplasmic reticulum (sect. V*A*). Although the purified ATPase restored basic properties attributable to the calcium transport system, the membranous vesicles reformed by the purified ATPase were incapable of sequestering Ca^{2+} in an ATP-dependent manner (198).

B. Reconstitution of Functioning Membrane Vesicles

Meissner and Fleischer (250, 251) attempted to find conditions under which membrane vesicles capable of sequestering Ca^{2+} could be reconstituted by deoxycholate-solubilized sarcoplasmic reticulum proteins and endogenous concentrations of lipid. They (250, 251) found that the reassembly of the components into vesicles by a slow removal of the detergent by dialysis at 20°C allowed optimal restoration of the ATP-supported accumulation of Ca^{2+}. Restoration of the Ca^{2+}-accumulating capacities measured in the absence and presence of oxalate was approximately 25 and 50%, respectively. Employing similar procedures, Repke et al. (307) obtained reconstituted vesicles whose storage capacity for calcium in the absence of oxalate was 70% that of original vesicles. Meissner and Fleischer (250) also showed that membrane vesicles were reformed by the ATPase preparation purified by their own procedures when a similar method was employed to reassemble the protein. The latter membrane vesicles, possessing a protein composition practically identical to that of the lighter of the two fractions (see sect. II*A*), were incapable of accumulating Ca^{2+} efficiently (250), indicating that the proper orientation of the ATPase molecules within the membrane is critical to the reconstitution of the functioning vesicles.

Reconstitution of functional vesicles from purified components was first achieved by Racker (299) and later by Warren et al. (385). Racker (299) admixed the ATPase protein purified by the method of MacLennan (198) with a large excess of soybean lipid in the presence of cholate. When the mixture was dialyzed in the presence of calcium-precipitable anions like

oxalate and P_i, the liposomal vesicles were found to incorporate the ATPase protein with simultaneous trapping of oxalate or P_i in the interior. Similar vesicles could be formed by sonication of the mixture, eliminating the use of cholate and a long period of dialysis (300, 305). These vesicles exhibited a remarkable Ca^{2+} accumulation in the presence of Mg^{2+} and ATP. Racker and co-workers (175, 177) showed that both phosphatidylcholine and phosphatidylethanolamine were required for full restoration of the calcium transport. However, in the reconstituted membranes of Racker and co-workers the preloading of the vesicles with calcium-precipitable anions was essential in restoring the activity, and the coupling ratio of the transport—i.e., the ratio of moles of Ca^{2+} transported for mole of ATP hydrolyzed (see sect. VI*B* and VII)—was much less than that of the original vesicles. MacLennan et al. (204), while confirming these findings, examined the possibility that incorporation of calsequestrin or the high-affinity calcium-binding protein into these vesicles could improve the coupling ratio. However, the coexistence of these additional components was found to be ineffective.

The reconstitution of vesicles capable of exhibiting a higher coupling ratio for calcium transport was achieved by Warren et al. (385), who employed an ATPase preparation whose lipid was more than 99% substituted by synthetic dioleyllecithin. This enzyme preparation possessed full ATPase activity. It was mixed with soybean phospholipids, and vesicles were formed by the dialysis method of Racker (299). The vesicles thus obtained exhibited Ca^{2+} accumulation as efficient as that of the intact vesicle, although the presence of oxalate on the interior was again needed to restore full transport activity.

These findings indicate that Ca^{2+}-dependent ATPase complexed with phospholipids possesses characteristics essential to the functional reconstitution of active calcium transport. However, these studies also indicate that the calcium transport activity of the reconstituted vesicles was not always restored to the level exhibited by the original sarcoplasmic reticulum vesicles. The incomplete restoration of activity might be due to either or both of the following: *a*) the reconstituted vesicles were not sufficiently sealed to permit an effective accumulation of Ca^{2+} (this state of the membrane is defined as being leaky); *b*) an additional minor component(s) essential for calcium transport was missing.

A heat-stable factor possessing the ability to increase the coupling ratio for the transport was found by Racker and Eytan (306) in the supernatant of mildly heated preparations of the purified ATPase of MacLennan (198). The properties of this factor resembled those of proteolipid extracted in acidified chloroform-methanol from the purified ATPase preparation by MacLennan (207). The structural and functional significance of this factor is unclear (sect. II*E*) and it remains to be seen whether this factor also represents an integral part of the active calcium transport system.

IV. MOLECULAR MODELS FOR ACTIVE TRANSPORT OF CATIONS

One of the chief goals of this article is to construct a realistic molecular

model, based on currently available experimental data, by which the mechanism of energy transduction during calcium transport by the sarcoplasmic reticulum can be comprehensively understood. Since Ca^{2+}-dependent ATPase complexed with phospholipids was shown in the previous section to form an essential part of the active calcium transport system, it is feasible to assume that the ATPase molecule may serve as an energy transducer as well as a translocator for Ca^{2+}. Before going into detail, therefore, it would be beneficial for the reader to become familiar with the idea that cation translocation may be associated with one or another type of molecular movement of the carrier protein during the process of the active cation transport. Several hypotheses for explaining the molecular mechanisms of active transport of cations like Na^+ and K^+, Ca^{2+}, and H^+ have been proposed. This section outlines characteristics of such models and considers the elementary steps and operational definition of the cation transport.

A. Shaw's Classical Model

In 1954 Shaw (98, 327) interpreted the mechanism of the active transport of Na^+ and K^+ across the cell membrane by suggesting that a cation-binding protein acted as a carrier (Fig. 1). This model, often referred to as the circulatory-carrier model, proved to be adequate to some extent in explaining the nature of the ion pumps in general and therefore provided a framework for the construction of more advanced models for active cation transport. It was proposed in this model that K^+ and Na^+ can only cross the membrane in combination with the carriers *X* and *Y*, *X* being K^+ specific and *Y* Na^+ specific. Further, *X* and *Y* are interconvertible and are in equilibrium at the outer surface of the membrane. At the inside surface, *X* is converted to *Y* with the expenditure of metabolic energy. This hypothesis provided a ready explanation for the vectorial properties of cation transport and thus became widely accepted as a prototype on which later models were based.

B. Proposed Major Models

In discord with the prediction by the Shaw's model, however, recent observations indicate that Na^+ and K^+ act cooperatively on the formation and decomposition of the intermediate of Na^+-K^+-dependent ATPase (367) and that the active transport of Na^+ and K^+ takes place only when both Na^+ and K^+ are bound to the carrier (94, 119). These findings indicated that the

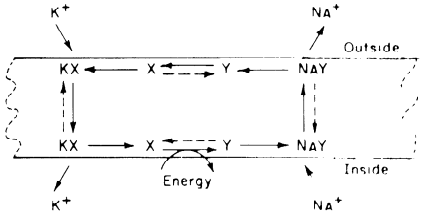


FIG. 1. Carrier hypothesis originally proposed by Shaw (327) to explain the active transport of Na^+ and K^+ across the erythrocyte membrane. [From Glynn (98).]

active transport of Na^+ and K^+ could not readily be explained by the simple circulatory-carrier model of Shaw. In the case of calcium transport by the sarcoplasmic reticulum, this model may be adequate at least in part in explaining the molecular mechanism of coupling between the ATPase reaction and the calcium transport (sect. VII and XII*A*). Prior to constructing a more advanced model for calcium transport in section XII, in this section we assess characteristics of three broad categories of molecular models that have been proposed so far.

1. Rotatory-carrier model

This first type of model assumes that cations bound to the carrier protein at either or both sides of the membranes are translocated to the other sides when the carrier molecule rotates within the membrane (Fig. 2*A*). When the translocation is completed, the carrier releases the cations due to a change in its affinity for cations. The protein then returns to the original state by a reverse rotation. This hypothesis, which may be referred to as the rotatory-carrier model, has been proposed by Yariv et al. (415a) and developed by Tonomura and Morales (371) and by Yamamoto and Tonomura (414) to explain the molecular mechanism of calcium transport by the sarcoplasmic reticulum membranes. A variant form of this model can be seen in Opit and Charnock's model for Na^+-K^+-dependent ATPase (273). This model enables us to visualize the functional and conformational transi-

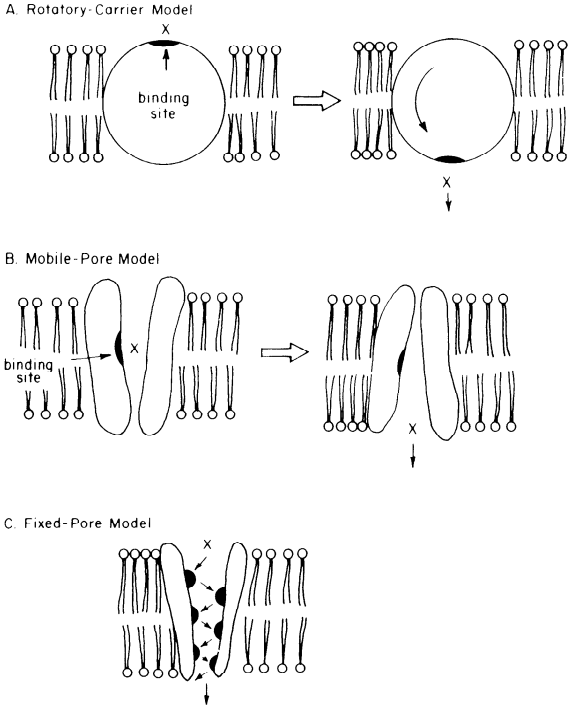


FIG. 2. Molecular models for active transport of cations. In each model, *X* represents cation to be translocated through the binding to a cation-binding site. *A*: rotatory-carrier model adopted by Tonomura and Morales (371) and Yamamoto and Tonomura (414). *B*: mobile-pore model adopted by Singer and coworkers (59, 330); Singer referred to this model as an aggregate rearrangement model (330). *C*: fixed-pore model proposed by Skou (333) and drawn by us according to his statements.

tions of the ATPase molecule that are assumed to take place continuously during the translocation of cations (see sect. XII). However, this model also assumes a thermodynamically unfavorable protein movement in which the hydrophilic group of the carrier protein capable of binding cations at the surface of the membrane has to move through the hydrophobic region of the lipid bilayer. The general problems arising from the rotatory motion of a polar group of a protein within the membrane were recently discussed by Singer (330).

2. *Mobile-pore model*

The second category of the molecular model may be referred to as the mobile-pore model, in which conformational changes within a protein-lined pore would help translocate cations across the membrane (Fig. 2*B*). The prototype of this model can be found in early models for Na^+-K^+-dependent ATPase proposed by Jardetzky (147) and Lowe (197). A more refined form of this type of model was proposed by Kyte (184), who presented evidence in accord with Singer's proposal (330). In this model, ion channels lined by protein are assumed to be open to the outside and closed to the inside of the membrane. Upon a conformational change of the protein complex induced by the energy expended by ATP hydrolysis, the channels open toward the inside of the membrane and at the same time the cations bound to the proteins become dissociated. The models proposed by Kyte (184) for Na^+-K^+-dependent ATPase and by Racker (301, 303), Martonosi et al. (230, 239), Shamoo and Goldstein (323a), and Dutton et al. (59) for Ca^{2+}-dependent ATPase fall into this category. This hypothesis is characterized by the assumption that during cation translocation a functional movement of the carrier molecule can take place while its hydrophilic group remains outside the membrane. However, this model is not always suitable for visually comprehending the continuous transition of conformational and functional properties of the ATPase molecule during the cation translocation (see sect. XII).

3. *Fixed-pore model*

The third category of the model is that proposed by Skou for the Na^+-K^+-dependent ATPase (332–334). This model, which also assumes a protein-lined pore across the membrane, may be referred to as the fixed-pore model because a large conformational change is not assumed to be necessary (Fig. 2*C*). This model is characterized by the assumption that cations are transferred by alterations in the affinities of carrier proteins for cations that are brought about by changes in electron distribution within the carrier protein. In this model, therefore, the affinities of the carrier protein for cations like Na^+ and K^+ presumably are different from one end to the other of the channel across the membrane, largely due to differences in charge distribution within the channel. This model remains to be substantiated by support-

ing evidence, since the true nature of so-called electron distribution that appears to control the affinity for cations remains unclear.

Thus, of these three categories of molecular model, the first two appear at the present stage to be more readily applicable, and therefore the usefulness of either of these in explaining the mechanism of calcium transport by the sarcoplasmic reticulum is discussed in section XII in light of the experimental evidence documented in this review.

C. Elementary Steps of Cation Transport

No matter what model one uses to explain the molecular mechanism of active cation transport, it is essential to correlate the alteration in affinity of the carrier protein for cations with some kind of movement or conformational change of the carrier molecule, a process not clearly understood at the present time. Pardee (284, 285) pointed out that during ion transport a carrier protein must play at least three roles in a successive manner: it has to recognize, translocate, and release ions. In initiating transport, a carrier protein has to bind the particular ion by recognizing it from among many kinds of solutes that exist within the system. This should be followed by the translocation of ions across the membrane, which presumably requires an energy expenditure to induce conformational alteration of the carrier protein. The affinity of the carrier for ions thus changes and ions are released from the carrier. The system then returns to its original state.

Assuming that Ca^{2+}-dependent ATPase serves as a carrier for translocating Ca^{2+} across the membranes of sarcoplasmic reticulum, the most important question to answer before constructing any molecular model would be: to which elementary step of the ATPase reaction, which presumably requires a thermodynamically dramatic change, does the translocation of Ca^{2+} correspond? Detailed accounts for this matter are described in sections VII and VIII.

D. Operational Definition for Cation Transport

It is appropriate to give here a more precise operational definition of the term *translocation* of cations in order to avoid confusion in analyzing the experimental data and in devising mechanisms of active calcium transport. When cations are incubated with the cation-transporting membranes, which usually form sealed vesicles, they are taken up by the membrane vesicles at the expense of energy, either by binding to the surface of the membrane or by actual uptake into the membrane vesicles. Under what conditions are these cations considered actually to be transported by the membrane? By operational definition, the cations would be considered to be translocated by the membrane when both of the following conditions are satisfied: *a*) the cations cannot be removed by the application, at one side of the membrane, of the highest possible concentration of a chelating agent, which by itself does not penetrate the membrane; *b*) the cations can be readily removed by the treatment of the membrane with cation-specific ionophores [X537A or

A23187 (375, 376)], detergents [Triton X-100 (159, 412)], or alkali (56) in the presence of a chelating agent. In the rotatory-carrier model, such conditions would correspond to an actual translocation of cations from one side of the membrane to the other, whereas in the pore models it could mean either an actual translocation of cations to the other side of the membrane or an apparent translocation due to trapping of cations inside the pocket formed by the carrier proteins.

V. CALCIUM PERMEABILITY AND BINDING OF CALCIUM BY MEMBRANE COMPONENTS

Distinctive features of the sarcoplasmic reticulum are that this membrane is simple in composition (sect. II) and that most of the components are assumed to function as parts of the only known physiological functions of this membrane: calcium transport and release. It therefore is appropriate to document our current knowledge of the calcium-binding properties of this membrane as well as its purified components. It will also be necessary to summarize major characteristics of the distribution and permeability of calcium in this membrane system.

A. Calcium Binding by Purified ATPase Preparation

Meissner and co-workers (245, 247) studied calcium binding by the purified ATPase by equilibrium dialysis and found that it was capable of binding 10–15 μmol of calcium/g of protein with a dissociation constant (K_D) of 0.5–2.5 μM under a range of pH and ionic conditions in the absence of ATP. The same preparation was found to form 6–7 μmol of phosphorylated intermediate/g of protein after incubation with ATP, an observation consistent with the earlier finding by MacLennan et al. (205). Assuming a molecular weight of 100,000 (sect. IIIA), the ATPase protein was estimated to contain 1–1.5 and 0.6–0.7 mol of calcium-binding and phosphorylation sites, respectively, per mole of ATPase, suggesting the existence of 2 mol of calcium-binding sites per mole of the active site of the ATPase. In fact, the molar ratio of these two sites was close to 2 over a fivefold range of the phosphoenzyme concentrations (247). Transient-state kinetic analysis also indicated that 2 mol of Ca^{2+} were bound to the ATPase, with a K_D of 0.3–0.5 μM, in a random sequence with ATP binding (159, 245; see also sect. VII). The equilibrium-dialysis studies by Ikemoto (127, 128) showed that at 0°C there were three classes of calcium-binding sites, one with higher affinity (K_D = 0.3 μM) denoted as α site, and two sites with lower affinity (K_D = 20 μM and 1 mM) denoted as β and γ sites, respectively. He found that at 22°C there were two α sites and no β site per mole of ATPase, whereas at 0°C there were one α and one β sites. The temperature-induced alteration in the numbers of high-affinity calcium-binding sites of the ATPase was in accord with the observation by Sumida and Tonomura (344) that the molar ratio of Ca^{2+} transported per ATP hydrolyzed decreased with decreasing temperature, becoming 1 at 0°C. These findings support the view that the high-

affinity calcium binding by the ATPase represents an essential part of the active calcium transport mechanism (see sect. VII and VIII).

B. Calcium Binding by Membranes of Sarcoplasmic Reticulum

Earlier observations that the membranes of sarcoplasmic reticulum possessed calcium-binding sites with high affinity (30, 36, 81) can probably be explained largely by the above-mentioned affinity of the purified ATPase for Ca^{2+}. Cohen and Selinger (36) measured by equilibrium centrifugation the calcium binding by the intact sarcoplasmic reticulum in the absence of ATP and suggested the existence of high-affinity calcium binding on the basis of the observation that about 10% of the total of 50 μmol bound/g of protein was not easily exchangeable at high salt concentrations. Its affinity for Ca^{2+} (K_D = 40 μM) was much lower than that found for the purified ATPase preparation, mentioned above. These observations probably represent an underestimate, either due to contaminating calcium in the preparation or to procedures that were not sensitive enough to detect the minute amount of the calcium binding. Indeed, Chevallier and Butow (30) redetermined by equilibrium dialysis the amount and affinity of calcium binding by sarcoplasmic reticulum vesicles whose contaminating calcium was thoroughly removed by passage through a chelating resin. They found that the sites with highest affinity bound about 10–20 μmol of calcium/g of protein with a K_D of 1.3 μM in the absence of ATP. A similar conclusion was reached by Fiehn and Migala (81), who found that the intact as well as the leaky membranes possessed a high intrinsic affinity for Ca^{2+} that did not depend on ATP. More recently, Miyamoto and Kasai (255) estimated the distribution of calcium bound to the outer and inner surfaces of the membranes of the sarcoplasmic reticulum and found that one of the two classes of calcium-binding sites on the outer surface exhibited a high affinity for Ca^{2+} (K_D * 1 μM) and bound about 12 μmol of calcium/g. Meissner et al. (247) compared the calcium binding by intact vesicles with that by the purified ATPase and found that the amounts and dissociation constants were indistinguishable. Taking 60–80% for the content of the 100,000-dalton ATPase in the total membrane protein (sect. II*C*), the site of high affinity was estimated to bind about 2 mol of calcium/mol of ATPase in the membrane. This membrane also exhibited calcium binding of much lower affinity (30, 81, 247, 255), which could be attributed at least partly to calsequestrin (see below).

The observed calcium binding with high affinity, presumably in the ATPase protein, might represent the initial reaction of the membrane with Ca^{2+}, as described in the previous section: the recognition of Ca^{2+} by the carrier protein. This was in accord with the findings of Yamada and Tonomura (408) and Ikemoto (128) that the affinity of this site for Ca^{2+} altered transiently during ATP hydrolysis (see sect. VII). It was not clear, however, what were the chemical properties of this calcium binding and what region of the protein was involved in the binding. Quite recently,

Shamoo and co-workers (326) reported that part of the ATPase protein was capable of acting as an ionophore for Ca^{2+} (see sect. XI*C*).

C. Calcium Binding by Extrinsic Proteins

The membranes of sarcoplasmic reticulum have been shown to contain another high-affinity calcium-binding protein (247, 274; see sect. II*F*). This protein bound 16–22 μmol of calcium/g of protein, or 1 mol of calcium/mol (55,000 daltons), and its K_D was 2.5–4 μM. The functional role of this protein in the active calcium transport is not yet known.

Calsequestrin is the second major constituent of the sarcoplasmic reticulum and exhibits calcium binding of much lower affinity and extremely high capacity. This protein, which was first found by MacLennan and Wong (206), bound 750–1000 μmol of calcium/g of protein (131, 132, 207, 247, 274). Since its molecular weight was reported to be between 44,000 and 65,000 (sect. II*F*), the calcium-binding capacity was estimated to amount to 35–60 mol/mol. Its affinity for Ca^{2+} was much lower than those of two other proteins already mentioned; the K_D of calsequestrin for Ca^{2+} was 1.3 mM (131) or 0.8 mM (274). Since this protein, possessing extremely high capacity and relatively low affinity for Ca^{2+}, was considered to be present on the interior of the vesicle (sect. II*F*), it was postulated to serve as major sites for calcium storage rather than participating directly in the active transport process (see also sect. III*B*). The low-affinity calcium binding found in the leaky membranes by Fiehn and Migala (81) and that found on the internal surface of the vesicle of intact sarcoplasmic reticulum in more recent studies by Miyamoto and Kasai (255) may at least partly be explained by the calcium binding by calsequestrin.

D. Distribution and Permeability of Cations in Sarcoplasmic Reticulum

1. Estimation of size of vesicular lumen and shell

In order to characterize the distribution of cations within the membrane vesicle and to examine the permeability of cations across this membrane, it is essential to estimate precisely the volumes of the internal lumen (space within the vesicle) and the shell (bilayer membrane forming the vesicle). This should help discriminate the outer and inner surfaces and the luminal water spaces of the vesicles and allow for the precise determination of the distribution of cations across the membrane.

Weber et al. (397) first reported the maximal vesicular volume of 8 ml/g of protein, based on the measurement of dry and wet weights after centrifugation. The true vesicular volume should be smaller, since the observed value did not exclude the intervesicular space. In fact, the vesicular volume was later estimated more precisely by Duggan and Martonosi (56), who

determined the excluded volume after incubation of the vesicles with membrane-impermeable agents such as [^{14}C]inulin and [^{14}C]dextran. They found a vesicular volume of 4–5 ml/g at pH 7.0. This observation was later reconfirmed by Miyamoto and Kasai (255), who obtained about 6.3 ml/g for [^{14}C]inulin exclusion volume. They also estimated the volume of the membrane shell to be 1.6 ml/g, based on the measurement of dry weight and density of the membrane. The calculated volume of the internal water space (4.7 ml/g) was incompatible with that estimated from calcium oxalate uptake (0.6–2 ml/g) or from a Scatchard plot analysis of distribution of Ca^{2+} (about 1.5 ml/g) (255). In the latter experiments, however, the existence of incompletely resealed vesicles within the preparation could lower the estimated value of the internal water space.

2. *Cation permeability of membrane vesicles*

In the absence of ATP, the membrane of sarcoplasmic reticulum is extremely impermeable to Ca^{2+}. Due to the low passive permeability to Ca^{2+}, it requires at least several hours to equilibrate the internal lumen of the vesicle with Ca^{2+} by the addition of a large amount of Ca^{2+} to the exterior of the membrane (148, 406; see sect. x). Duggan and Martonosi (56) observed that [^{14}C]inulin (5000 daltons) and [^{14}C]dextran (15,000–90,000 daltons) were excluded by the vesicular volume at pH 7–9. However, when the vesicles were treated with EDTA or EGTA at an alkaline pH (8–9), the [^{14}C]inulin-inaccessible water space became smaller, indicating that this molecule of 5000 mol wt penetrated the membrane. The decrease in the inulin-inaccessible space was paralleled by the increase in the permeability of the membrane to Ca^{2+} and Mg^{2+}. They concluded that the membrane became more leaky in the presence of EGTA or EDTA at alkaline pH and suggested that the increase in the membrane permeability was a dual function of the content of membrane-bound cations and H^+ concentration.

The observed permeability of the sarcoplasmic reticulum membranes to divalent cations was considered to be derived largely from the inherent properties of phospholipid bilayer membranes, which are extremely impermeable to cations (41). Jilka et al. (148) reported that the passive permeability of Ca^{2+} in liposomes, made of lecithin or phospholipids of sarcoplasmic reticulum, increased when increasing amounts of the ATPase protein were added to reconstitute the membrane, becoming 10^4- to 10^6-fold greater than that of the original liposomes. The influx of Ca^{2+} across the native membranes during ATP-supported uptake of Ca^{2+} was estimated to be 50–500 times larger than the passive permeability of the same membrane observed in the absence of ATP (148).

Calcium permeability of sarcoplasmic reticulum was found to change with varying Ca^{2+} concentration outside and inside the vesicles, Ca^o and Ca^i, respectively. Under conditions where Ca^i was held constant by the presence of oxalate (see sect. v, *D3*), the calcium efflux increased remarkably immediately after an elevation of Ca^o (110, 398). Makinose (214) reported that elevation of Ca^o evoked the spontaneous release of a portion of the

previously accumulated calcium with simultaneous uptake of the added Ca^o. Katz et al. (160, 163) observed that increasing Ca^i at any level of Ca^o caused little or no effect on the calcium efflux, so that the calcium permeability coefficients (calcium efflux/Ca^i) increased with decrease in Ca^i. From these findings Katz et al. (163) suggested that the passive calcium permeability of the membrane of sarcoplasmic reticulum increased when Ca^o was increased and decreased when Ca^i was increased.

3. *Estimation of ionized and bound calcium within vesicles*

Since the precise determination of localization and relative proportion of ionized and bound calcium within sarcoplasmic reticulum vesicles is essential in understanding the molecular mechanism of active transport of calcium across the membrane, many investigations paid attention to the localization of ionized and bound calcium within the sarcoplasmic reticulum when its function was energized by ATP with or without addition of precipitating anions like oxalate.

In the absence of oxalate, the ATP-supported maximal filling capacity of calcium within the vesicle is as high as 150 μmol/g of protein (65, 106, 270, 392, 398). If the intravesicular water space is taken to be between 1 and 5 ml/g (see sect. v, *D1*), the average Ca^{2+} concentration within the vesicular space would be 30–150 mM, assuming that all the calcium was ionized. However, the concentration of Ca^{2+} within the vesicles during oxalate-facilitated transport of calcium (see below) was calculated by Weber et al. (398) and Ogawa (270) to be as high as 0.5 mM, employing 2×10^{-9} (106) and 2×10^{-7} (270) M^2, respectively, for solubility products of calcium oxalate. These results suggested that in the absence of oxalate most of the calcium is bound to the membrane, leaving only a small amount as the ionized form. It is not clear which component(s) binds such an amount of calcium (150 μmol/g of protein at the highest). Calsequestrin could serve as major sequestering sites for calcium, although no direct evidence exists. By simple calculation, a large part (60–120 μmol/g) can be assumed to associate with this protein, whose capacity for calcium was estimated to be 35–60 mol/mol (assuming its content of 10% of the total protein).

When oxalate was included in the medium, the amount of calcium taken up by the vesicle increased to as much as 8–10 mmol/g of protein (106, 398). Since an equimolar content of calcium and oxalate was found under these conditions, Hasselbach (106) and Weber et al. (398) concluded that calcium oxalate was formed within the vesicle. This complex was visualized as crystals by electron microscopy (53, 106). These results indicate that oxalate, freely permeable to the membrane, was capable of maintaining an intravesicular free Ca^{2+} concentration and accumulating more calcium within the vesicle by forming a complex with calcium.

4. *Exchangeability of cations in sarcoplasmic reticulum*

It is of interest to consider whether the accumulation of the large

amount of calcium within the vesicle is accompanied by release of counterions from the inside to the outside of the membrane (25–27), although permeability of sarcoplasmic reticulum to ions other than Ca^{2+} has not been determined. A. P. Carvalho and Leo (25, 27) found that sarcoplasmic reticulum possessed a fixed cation-binding capacity of about 350 μeq/g of protein at neutral pH and that, when the vesicles took up calcium in the presence of ATP, cations (such as Mg^{2+}, K^+, and H^+) were released in amounts equivalent to the calcium taken up. Since most of the cation lost from the vesicles was Mg^{2+}, this cation might have acted as a counterion for Ca^{2+}. In devising the most probable mechanism in which Mg^{2+} serves as a counterion for Ca^{2+}, one has to assume that the membrane of sarcoplasmic reticulum is permeable to Mg^{2+}. However, in recent studies by T. Kometani and M. Kasai (personal communication), permeability of the sarcoplasmic reticulum membrane to Mg^{2+} was estimated to be only 3 times greater than permeability to Ca^{2+}. In their experiment, in which cation permeability was estimated by measuring the change in light-scattering intensity after addition of salt, it is unclear whether this method would be sufficient enough to detect permeability of Mg^{2+} and Ca^{2+}, which are prone to bind tightly to the membrane. The exact nature of the Mg^{2+} permeability of sarcoplasmic reticulum and its role in the active calcium transport remain to be elucidated.

VI. GENERAL PROPERTIES OF ATP HYDROLYSIS BY SARCOPLASMIC RETICULUM

Active transport of Ca^{2+} across the membranes of sarcoplasmic reticulum is an energy-requiring process. The Ca^{2+}-dependent ATP hydrolysis by this membrane is considered to represent an energy source for this process, through which the translocation of Ca^{2+} against a concentration gradient can be achieved effectively. In this section, therefore, we describe the properties of the overall reaction of the ATP hydrolysis by sarcoplasmic reticulum. These properties provide a basic framework for understanding the mechanism coupling the ATPase reaction with calcium transport, as discussed in the next two sections.

A. Basic and Extra ATP Splitting by Sarcoplasmic Reticulum

The membrane of sarcoplasmic reticulum exhibits two types of ATP hydrolysis, Ca^{2+}-independent and Ca^{2+}-dependent ATPase activities (111, 112, 215). Although both require Mg^{2+} for full activation, the former, also termed the *basic* ATPase, is independent of Ca^{2+}, whereas the latter, termed the *extra* ATPase, is absolutely dependent on Ca^{2+} concentrations between 0.01 and 1 μM. Specificity of the basic ATPase for nucleotide triphosphates is lower (217), and the K_m for ATP is much higher (398, 412) than those of the Ca^{2+}-dependent ATPase. These activities have different temperature coefficients (139, 412) and pH (412) profiles. The Ca^{2+}-dependent ATPase is inhibited markedly by thiol reagents, whereas the basic ATPase is insensi-

tive to these agents (111, 114, 140, 258, 412). The basic ATPase activity is abolished when the sarcoplasmic reticulum membrane is solubilized by detergents (243, 383). It is uncertain at present whether the two activities are attributable to different enzymes (11, 18, 223, 321, 374) or whether they represent different manifestation of the same enzyme, which can be interconverted depending on the conformation of protein or the membrane structure. The latter possibility was suggested by Inesi et al. (139), who recently reported that the basic ATPase was present predominantly in the lighter fraction (see sect. IIA) of the sarcoplasmic reticulum preparations. Froehlich and Taylor (89) suggested the same possibility, based on their studies of the transient-state kinetic properties of this ATPase.

B. Coupling of ATP Hydrolysis with Calcium Transport

Immediately after the addition of Ca^{2+} to the reaction medium, the rate of ATP hydrolysis is stimulated markedly, becoming 10–30 times greater than the basic activity rate (106, 397). The Ca^{2+}-activated portion of the ATP hydrolysis, which was termed "the extra ATP splitting" by Hasselbach and Makinose (111), was found to be coupled with uptake of Ca^{2+} by the vesicles (106, 111, 113). When Ca^{2+} concentration in the medium was reduced to less than 0.01 μM, due to calcium uptake by the vesicles, the rate of ATP hydrolysis returned to the original level. Treatment of the vesicles with low concentrations of detergents (243, 383), diethylether (79, 140), or EGTA at alkaline pH (56) induced "leakiness" of the membrane and prevented net uptake of Ca^{2+}. Under these conditions, the high rate of the Ca^{2+}-dependent ATP hydrolysis was maintained for prolonged period of time. Thus, the Ca^{2+}-dependent ATPase activity is closely related to the accumulation of Ca^{2+} by the membrane vesicles. In the intact vesicles, a tight coupling was found to exist between the two, giving 2 mol of Ca^{2+} transported for 1 mol of ATP hydrolyzed under a variety of conditions in the presence of oxalate (106, 111, 113, 236). Weber et al. (392, 398) observed that the stoichiometric coupling of 2 mol of Ca^{2+} taken up for each mole of ATP hydrolyzed in the absence of oxalate was maintained over a wide range of ATP concentrations. Comparing the initial rates of ATP hydrolysis and uptake of ^{45}Ca, Yamada et al. (411) found that the coupling ratio of 2 was strictly maintained during the initial phase of the reaction in the absence of oxalate. Essentially similar results were obtained by Kurzmack and Inesi (181), who measured the initial rates of ATP hydrolysis and absorption changes in the presence of murexide, used to monitor Ca^{2+} concentration. The molecular mechanism of this coupling is detailed in section VIII.

C. Substrate Specificity

The Ca^{2+}-dependent ATPase exhibits extremely high affinity for ATP, with an apparent K_m of several micromoles per liter (381, 398, 412). This enzyme also catalyzes hydrolysis of other natural nucleoside triphosphates

(24, 210, 217, 235). The velocity of hydrolysis is graded in the following sequence: ATP (1.0) > ITP (0.8) > GTP (0.7) > CTP (0.55) > UTP (0.25) [numbers in parentheses represent velocities of hydrolysis relative to that of ATP (217)]. Furthermore, other phosphate compounds, such as acetylphosphate (43, 44, 52, 86, 205, 298), carbamylphosphate (298), and *p*-nitrophenylphosphate (135), were shown to serve as substrates, although the rates of their hydrolysis are extremely low. Active calcium transport is induced by all of these analogues and phosphate compounds, and the coupling of 2 mol of Ca^{2+} for each mole of phosphate liberated is strictly maintained (86, 135, 217).

D. Effects of Cations and pH

The Ca^{2+}-dependent hydrolysis of ATP by sarcoplasmic reticulum 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; see below), the rate of ATP hydrolysis rises with increasing Ca^{2+} concentrations, reaching maximal velocity at about 1 μM Ca^{2+} (113, 215, 217). Half-maximal activation is seen at 0.3–0.5 μM Ca^{2+} (398, 412). The Hill coefficient for activation by Ca^{2+} is nearly 2 (356, 381), which is consistent with the observation that the molar ratio of coupling between ATP hydrolysis and calcium transport is 2 (see sect. VI*B*). At concentrations above 0.1 mM, Ca^{2+} was found to inhibit ATP hydrolysis (see sect. VII).

Although its affinity is about 1/80 that of Ca^{2+}, Sr^{2+} could substitute for Ca^{2+} (252, 398, 408). Also, Ca^{2+} could be replaced by Co^{2+} (198). The trivalent cation La^{3+}, which exhibits an electrostatic attraction greater than Ca^{2+} for any negatively charged binding site, at relatively high concentrations (about 10 μM) inhibits both ATP hydrolysis and calcium transport (30, 179, 408). Another report indicates that La^{3+} is not effective in inhibiting ATPase activity (75).

It was found that Mg^{2+} has at least two important roles in ATP hydrolysis by the sarcoplasmic reticulum. One is to accelerate the decomposition of the phosphorylated intermediate formed during the reaction; this reaction is detailed in section VII*D*. The other is to form an equimolar complex with ATP and serve as the true substrate for the Ca^{2+}-dependent ATPase (381, 398, 412). The latter effect of Mg^{2+} was implied by the findings that Mg^{2+} concentration in excess over that of ATP gave optimal activity and that Ca^{2+}-dependency profile of the enzyme activity was not altered within a broad range of Mg^{2+} concentration (398, 412). More direct evidence was presented by Vianna (381), who 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 concentration of MgATP complex. Weber et al. (398) showed that the CaATP complex did not serve as substrate. The Mg^{2+} can be replaced by Co^{2+} or Mn^{2+}, but not by Zn^{2+} (204, 408).

The Ca^{2+}-dependent ATPase activity of sarcoplasmic reticulum is inhibited by Na^+ and K^+ (45, 46, 84, 97, 329, 357). Inhibition was significant at high concentration of the monovalent cations (> 0.2 M), but only in the presence of low concentration of ATP and Ca^{2+}.

The dependence of the Ca^{2+}-dependent ATPase activity on pH exhibits a bell-shaped profile, with the optimal activity at pH 6.5–7.5. The decrease in enzyme activity was found to be more profound at alkaline pH.

E. Formation of Phosphoprotein as Reaction Intermediate of ATPase

The membranes of sarcoplasmic reticulum were found to catalyze phosphate exchange between ADP and ATP in the presence of Ca^{2+} and Mg^{2+} (67, 112, 113). On the basis of observations that this rapid exchange of phosphate exhibited the same dependence on Ca^{2+} concentration as did ATP hydrolysis and calcium uptake by the same membranes, Hasselbach and Makinose (106, 111) postulated that a high-energy phosphoprotein was formed as the intermediate of the reaction during active transport of Ca^{2+}. Makinose (210) showed that the sarcoplasmic reticulum transferred the terminal phosphate of ITP or GTP to ADP and the terminal phosphate of ATP not only to ADP but also to IDP. Yamamoto and Tonomura (412, 413) demonstrated that a protein of sarcoplasmic reticulum was phosphorylated when the Ca^{2+}-dependent reaction of this membrane with [γ-^{32}P]ATP was quenched by TCA. Similar observations were made independently by Makinose (211), and later confirmed by Martonosi (224, 227) and Inesi and co-workers (137, 142). The 1 mol of phosphate incorporated was found to correspond to 1 mol of the ATPase protein when its molecular weight later was clarified (205). Phosphoprotein levels, at the steady state, depend on the concentration of Ca^{2+} and ATP and parallel those of the Ca^{2+}-dependent ATPase activity. The ratio at steady state of ATPase activity to the concentration of the phosphoprotein, v_o/[EP], decreases with an increase in pH within the alkaline pH range. This pH profile was quite similar to that of the Ca^{2+}-dependent ATPase activity (413). These results suggest that the phosphorylated protein is a true intermediate of the Ca^{2+}-dependent ATPase reaction, although this assumption was not proven until the elementary steps of the reaction were analyzed extensively (see sect. VII).

The phosphoprotein isolated after quenching by TCA is stable at acidic pH, but is extremely unstable at alkaline pH (211, 412, 413). It is hydrolyzed by treatment with hydroxylamine (211, 412, 415), resulting in the formation of hydroxamate (415). These stability characteristics indicate that this phosphoprotein is similar to the acylphosphoprotein intermediate of Na^+-K^+-dependent ATPase, reported by Post and co-workers (292, 294, 295) and Skou (334). Degani and Boyer (42) reported that the reductive cleavage of the acylphosphate bond of the phosphoprotein by NH_4Br yielded homoserine after acid hydrolysis of the protein, indicating that the phosphoryl group was covalently bound to the β-carboxyl group of aspartate. Bastide et al.

(14) examined the chemical and electrophoretic properties of ^{32}P-labeled phosphoryl peptide produced after proteolysis of phosphorylated intermediates of Ca^{2+}-dependent ATPase of sarcoplasmic reticulum and Na^+-K^+-dependent ATPase of kidney microsomes and found that the probable active site tripeptide sequence for both ATPases was (Ser or Thr)-Asp-Lys, suggesting that the peptide structure of the active site region of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum was similar to that of Na^+-K^+-dependent ATPase (293). Recently the tripeptide sequence was confirmed by Allen and Green (3), who isolated a tryptic peptide that contained 31 residues from the active site of the Ca^{2+}-dependent ATPase and partially determined its sequence. These observations elucidated chemical characteristics of the phosphoprotein isolated after quenching by TCA, but it is not certain whether these properties represent the inherent nature of a phosphoryl protein in its intact form.

F. Chemical Modification of Ca^{2+}-Dependent ATPase

The Ca^{2+}-dependent ATPase activity is irreversibly inactivated by mercurials or alkylating agents (111, 114, 235, 258, 279, 280, 325), suggesting that thiol groups are essential in maintaining the enzyme activity. This blockade was prevented completely by ATP through protection of 1 or 2 mol of essential thiol group(s) per mole of this enzyme (102, 114, 279, 280). Based on kinetic analysis of the inactivation profile of Ca^{2+}-dependent ATPase induced by 5,5′-dithiobis(2-nitrobenzoate), Murphy (258) suggested that Ca^{2+} produced a marked change in reactivity of the essential thiol group with this compound. On the basis of observations that the photooxidation of sarcoplasmic reticulum with methylene blue or rose bengal resulted in inactivation of both ATPase and calcium uptake, Yu et al. (416–418) suggested that histidine residues also maintain the Ca^{2+}-dependent ATPase activity. Similar results were obtained by Martonosi and co-workers (35, 232). These findings are consistent with the recent observation by Tenu et al. (355), who reported that ethoxyformylation of 1 or 2 mol of histidine residue(s) of ATPase was accompanied by the complete loss of Ca^{2+}-dependent ATPase activity.

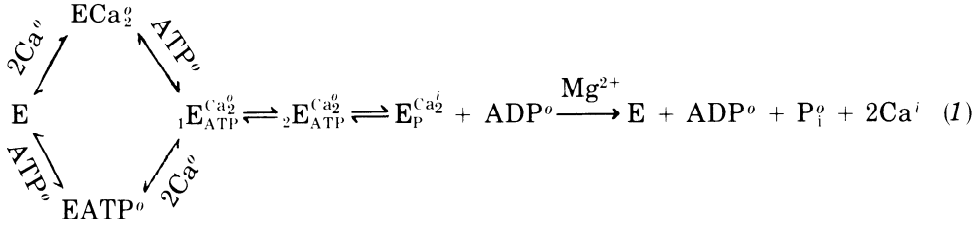
VII. ELEMENTARY STEPS OF ADENOSINETRIPHOSPHATASE AND CHANGE IN AFFINITY FOR CATIONS

In this section we document the molecular mechanism of coupling between the elementary steps of the ATPase reaction and the three major steps of the ion transport—i.e., recognition, translocation, and release of calcium ions (sect. IV)—during the process of calcium transport across the membrane of sarcoplasmic reticulum. In order to understand the coupling mechanism, kinetic analyses of partial reactions constituting the overall ATPase reaction should be a prime necessity. For accurate analysis of these elementary steps and their comparison with the elementary steps of calcium transport, it is also essential to consider the vectorial properties of the actions of this enzyme; localization of the sites of action of substrates,

products, and permeates should always be distinguished during the active transport of cations. In this regard, intact vesicles are much more suitable than the purified ATPase preparation or reconstituted vesicles, since in the former the coupling of the calcium transport is tighter (see sect. I and VI) and the sidedness of the membrane is better maintained (sect. II and IV).

A. Elementary Steps During ATPase Reaction

Based on the kinetic analysis of the partial and overall reactions of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum, the following reaction scheme was proposed by Kanazawa et al. (159) to represent the mechanism of coupling between the ATPase and calcium transport:



where *i* and *o* indicate the inside and outside of the membrane vesicles, respectively. Two moles of Ca^{2+} and 1 mol of ATP bind in a random sequence to 1 mol of the ATPase, E, at the outer surface of the membrane, forming the first Michaelis complex, $_1E_{ATP}^{Ca_2^o}$. This site is followed by formation of a second complex, $_2E_{ATP}^{Ca_2^o}$ (evidence supporting the presence of the two kinds of Michaelis complex is documented in sect. IX*A*). Calcium is subsequently translocated from outside to inside the membrane when the phosphorylated intermediate $E_P^{Ca_2^i}$ is formed. Calcium is then released from the enzyme into the interior of the vesicle, with simultaneous decomposition of the phosphorylated intermediate into the products ADP and P_i, both of which are liberated from the enzyme to the exterior of the vesicle.

In analyzing the translocation of cations across the membrane, it is important to define clearly the inside and outside of the membrane through which cations are translocated. Operationally, radioactive substances and cations are considered to exist outside the membrane when they are readily removed by washing with a neutral solution containing unlabeled substances and a chelating agent. On the other hand, radioactive substances and cations are considered to exist within the membrane vesicle when they are not removed by simple washing with or without unlabeled substances and/or a chelating agent, but are removed by unlabeled substances and a chelating agent after treatment with detergents or at alkaline pH to induce leakiness of the membrane (see sect. V, *D2*).

B. Formation of Phosphorylated Intermediate EP from E and ATP

The initial step in calcium transport, the recognition of Ca^{2+}, would presumably correspond to the complex formation of the ATPase with Ca^{2+}

and ATP in the scheme shown above. These processes, occurring in a random sequence, are very rapid reactions that are followed by the formation of the phosphorylated intermediate.

Immediately after the addition of ATP to sarcoplasmic reticulum in the presence of Ca^{2+}, the phosphorylated intermediate EP is rapidly formed, reaching maximal level within 1 s at 15°C. The liberation of P_i exhibits an initial lag phase (159), which coincides with the rapid increase in EP formation. The observed lag phase of P_i liberation can be explained by calculations based on the amount and rate of EP formation and the rate of its turnover, assuming that all of the P_i is liberated from EP. These observations indicate that EP is the true intermediate of the ATPase reaction.

Based on an examination of the dependence of the velocity of EP formation, v_f, on the concentrations of ATP and Ca^{2+}, the following equation was obtained (159):

$$v_f = \frac{V_f}{\left\{1 + \left(\frac{K_{Ca}}{[Ca]}\right)^2\right\}\left\{1 + \frac{K_m}{[ATP]}\right\}} \qquad (2)$$

where V_f, K_{Ca}, and K_m represent the maximal velocity of formation of EP, and concentrations of Ca^{2+} and ATP that give the half-maximal velocity of EP formation, respectively. These findings indicate that 2 mol of Ca^{2+} and 1 mol of ATP are bound to 1 mol of ATPase in a random sequence to form the first Michaelis complex, ${}_1E_{ATP}^{Ca_2}$.

Absence of a lag phase in EP formation, even in the presence of an extremely low concentration of ATP, suggests that the steps prior to the EP formation, i.e., the formation of the Michaelis complex, occur very rapidly at the outer surface of the membrane. Formation of $E^{32}P$ can be terminated immediately if EGTA is added to remove Ca^{2+} in the medium or if a large amount of unlabeled ATP is added to dilute the radioactive ATP. Since the membranes of sarcoplasmic reticulum are impermeable to ATP and EGTA (398), these findings indicate that Ca^{2+} and ATP interact with the ATPase enzyme to form the complex ${}_1E_{ATP}^{Ca_2}$ at the outer surface of the membrane.

Most divalent cations, with the exception of Sr^{2+}, are incapable of substituting for Ca^{2+} to form EP. High concentrations of Mg^{2+} inhibit EP formation by competing with Ca^{2+}. Yamada and Tonomura (408) determined kinetically the affinities of the Ca^{2+}-dependent ATPase for these divalent cations and found K_D values of 0.3 μM, 27 μM, and about 9 mM for Ca^{2+}, Sr^{2+}, and Mg^{2+}, respectively. The observed value of K_D for Ca^{2+}, which was independent of the presence of ATP, is of the same order of magnitude as that obtained by Chevallier and Butow (30) and Meissner et al. (247) with the equilibrium-dialysis method (see sect. v*A*). The latter method also gave the same number of calcium-binding sites (nearly 2 mol/mol of ATPase) as did kinetic analysis.

C. Formation of ATP from EP and ADP

Evidence indicating that the translocation of Ca^{2+} across the membrane is coupled to EP formation was obtained by Kanazawa et al. (158, 159), who performed a kinetic analysis of the reverse reaction of the ATPase, where ATP is formed from EP and ADP. When a large amount of EGTA was added to remove Ca^{2+} from the medium after the completion of EP formation, further formation of EP was instantaneously terminated. This was accompanied by the liberation of P_i from EP and the P_i liberation followed first-order kinetics. However, when ADP was added simultaneously with EGTA, the decrease in EP was not accompanied by the liberation of P_i. Analysis of the product on thin-layer chromatography showed that ATP was formed in an amount equal to that of the EP that existed before the addition of ADP and EGTA. In a similar experiment at high pH, where EP decomposition is markedly slowed, addition of ADP without EGTA resulted in a substantial, but not complete, conversion of EP to ATP. These findings suggested that ${}_2E^{Ca_2^o}_{ATP}$ and $E_P^{Ca_2^i}$ + ADP was completely reversible. The equilibrium of the latter step (EP + ADP $\rightleftharpoons$ E·ATP) favors the formation of E·ATP in the presence of a large amount of ADP.

The reverse reaction did not require Ca^{2+} in the medium (above), in contrast to the forward reaction that absolutely requires Ca^{2+}. Thus, external Ca^{2+}, for which there was an absolute requirement in the forward reaction, does not participate in the complete backward reaction. In view of the reversibility of this reaction step, it was possible, however, that Ca^{2+} ions inside the vesicle *did* participate in the reverse reaction:

$$E + ATP \underset{Ca^i}{\overset{Ca^o}{\rightleftharpoons}} EP + ADP \qquad (3)$$

Evidence in support of the participation of intravesicular Ca^{2+} in the reverse reaction was obtained in the following experiments. Sarcoplasmic reticulum was phosphorylated by [γ-^{32}P]ATP in the presence of Ca^{2+} at alkaline pH, where the membrane was leaky. At timed intervals after $E^{32}P$ formation was halted by EGTA, ADP was added and the amount of $E^{32}P$ decay was examined. The amount of $E^{32}P$ that decayed in response to the added ADP decreased with time. This experiment was interpreted to mean that the depletion of the intravesicular Ca^{2+} abolished the reverse reaction — that is, the reaction of EP with ADP to form ATP. When sarcoplasmic reticulum was solubilized with Triton X-100, the addition of EGTA and ADP after $E^{32}P$ formation did not induce the formation of [γ-^{32}P]ATP, while the simultaneous addition of unlabeled ATP and ADP induced the reverse reaction. These findings indicated that intravesicular Ca^{2+} was required to form ATP from EP and ADP.

D. Decomposition of EP

When calcium is translocated from outside to inside the membrane, it has to be released from the enzyme into the vesicular lumen. This process is related to the decomposition of EP, which can be measured by either of two different methods. One is to measure the ratio v_o/[EP], i.e., the rate of ATPase activity per unit of concentration of EP at steady state. The other is to measure directly the decomposition of $E^{32}P$ after its formation is terminated by the addition of EGTA to remove Ca^{2+} or of unlabeled ATP to dilute the radioactive ATP and thus halt ^{32}P incorporation into the enzyme. The time course of the EP decomposition was found to follow first-order reaction kinetics and not to exhibit a lag phase. Since the membrane of sarcoplasmic reticulum is impermeable to EGTA and ATP (see above), these findings suggested that Ca^{2+} and ATP reacted with the ATPase at the outer surface of the membrane, as stated in section VII*B*. Because the equilibrium between E + ATP and EP + ADP favors the formation of EP, unless a large amount of ADP is present, the ratio v_o/[EP] is equal to the specific rate constant, k_d, of the decomposition of EP.

Inesi et al. (142), Martonosi (227), Kanazawa et al. (159), and Panet and Selinger (279) found that the decomposition of EP required Mg^{2+}. It is important to know whether Mg^{2+} causes EP to decompose outside or inside of the membrane vesicle. When Mg^{2+} was removed by EDTA after the EP formation, the decomposition of EP did not stop immediately, but several seconds elapsed before the decomposition was completely prevented (159). When the membrane was solubilized with detergents, however, the addition of EDTA instantaneously terminated the decomposition of EP. These observations suggest that the membranes of sarcoplasmic reticulum are more permeable to Mg^{2+} than Ca^{2+} and that Mg^{2+} may participate in EP decomposition at the interior of the vesicle. Although the permeability of the membrane to Mg^{2+} was suggested to be greater than that to Ca^{2+} (sect. v, *D4*), a more precise quantitative determination must still be made. Therefore, the suggested role for Mg^{2+} in promoting EP decomposition cannot be clarified until the Mg^{2+} permeability of the membrane becomes more precisely known.

Employing deoxycholate-treated membranes of sarcoplasmic reticulum, in which the inside and outside of the membranes are kinetically indistinguishable, Yamada and Tonomura (408) measured the dependence of v_o/[EP] on the concentration of both Mg^{2+} and Ca^{2+} and found that Ca^{2+} inhibited the decomposition of EP by competing at the site where Mg^{2+} accelerated decomposition of EP. Under these conditions, the ratio of affinity of EP for Ca^{2+} to that for Mg^{2+} was 2.5:1. These findings indicated that the phosphorylated enzyme scarcely distinguishes between the two divalent cations, the affinities for both being of a comparable order. In contrast, the affinity of the unphosphorylated enzyme for Ca^{2+} is about 30,000 times higher than that for Mg^{2+} (see sect. VII*B*). Yamada and Tonomura (408) thus concluded that the formation of EP that coincided with the translocation of Ca^{2+} across the

membrane is accompanied by a dramatic change in the affinity of the enzyme for Ca^{2+} and Mg^{2+}.

Employing sarcoplasmic reticulum vesicles whose exchangeable Mg^{2+} was thoroughly removed by washing with *trans*-1,2-diaminocyclohexanetetraacetic acid (CDTA), Garrahan et al. (96) examined the effects of Mg^{2+} on EP decomposition. They showed that EP formed in such membranes in the absence of added Mg^{2+} did not decompose when a large amount of Mg^{2+} was added subsequently, whereas decomposition of EP formed in the presence of added Mg^{2+} was not prevented when Mg^{2+} was removed by the addition of CDTA. These authors also obtained essentially similar findings in the sarcoplasmic reticulum solubilized with Triton X-100. Based on these observations, Garrahan et al. (96) suggested that the calcium-binding sites required for the formation of EP were distinct from the magnesium-binding site(s) required for the EP decomposition and that the chelating agent was incapable of removing magnesium that was bound to EP. These observations, however, did not exclude the possibility that CDTA treatment of the membranes altered the kinetic properties of EP so that the Mg^{2+} required for EP decomposition became inexchangeable. In fact, the filling capacity for Ca^{2+} and the amount of EP formation in the CDTA-treated vesicles were less than 10% and 20%, respectively, of those of the control vesicles. Alternatively, the decomposition of EP observed by Garrahan et al. (96) may have required both binding of Mg^{2+} to EP, which was not removed by CDTA, and binding to a site at which Mg^{2+} competed with Ca^{2+}. Bound, but not free, magnesium was also reported to be required for the normal decomposition of EP in Na^+-K^+-dependent ATPase (Y. Fukushima and R. L. Post, personal communication). The exact role of Mg^{2+} in controlling the decomposition of EP remains to be elucidated.

E. Reaction Mechanism of Hydrolysis of p-Nitrophenylphosphate

The sarcoplasmic reticulum can catalyze hydrolysis of *p*-nitrophenylphosphate (*p*NPP), a low-energy phosphate compound, which can induce active calcium transport, with the coupling of 2 mol of Ca^{2+} for each mole of *p*-nitrophenyl (*p*NP) liberated (135). Since the extremely low rate of its hydrolysis may permit more precise determination of the rate constants of elementary steps of the active calcium transport, Nakamura and Tonomura (266a) investigated kinetic properties of the formation and decomposition of the reaction intermediate during hydrolysis of this compound. When sarcoplasmic reticulum was incubated with [^{32}P]*p*-nitrophenylphosphate (*p*N^{32}P) in the presence of a high concentration of Ca^{2+} (10 mM) at alkaline pH, TCA-stable phosphoprotein was formed. The rate of formation of this phosphoprotein was extremely low, while its amount was almost equal to the amount of EP formed from E and ATP. Stability characteristics of the phosphoprotein intermediate were essentially similar to EP in the ATPase reaction. In addition, ATP was formed stoichiometrically on incubation of the former phosphoprotein with ADP, indicating that it is kinetically

indistinguishable from EP. The phosphoprotein intermediate formed during the hydrolysis of *p*NPP thus was referred to as EP.

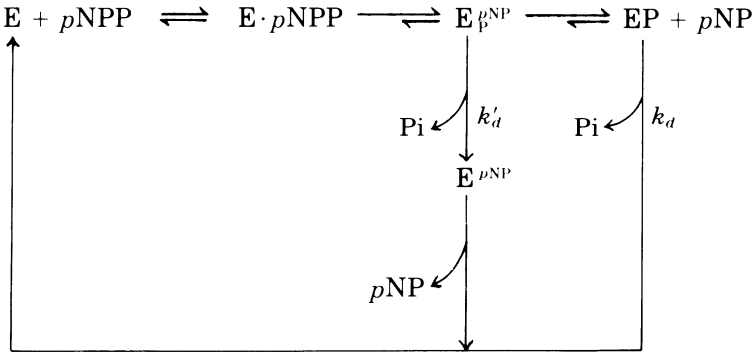
Measurement of decay in EP (formed from *p*NPP), after further formation of $E^{32}P$ was stopped by the addition of unlabeled *p*NPP, exhibited biphasic time courses. Although the fast phase was independent of divalent cations, the rate of the slow phase increased with increasing Mg^{2+} concentration and was competitively inhibited by Ca^{2+}.

Based on these findings, Nakamura and Tonomura (266a) suggested the existence of two types of phosphoprotein intermediates with and without bound *p*NP (E_P^{pNP} and EP) and assumed that the decay constant (k_d') of the former is much larger than that (k_d) of the latter. The rate of *p*NP liberation at the steady state was much smaller than the sum of $k_d'[E_P^{pNP}]$ and $k_d[EP]$. Thus, the fast-decomposing intermediate was interpreted to be derived from the reaction,

$$E_P^{pNP} \rightarrow E^{pNP} + P_i \rightarrow E + pNP$$

Such an assumption was consistent with the finding that the amount of the initial burst of *p*NP after the reaction with TCA was stopped was higher than the amount of EP.

The following mechanism was proposed to explain these findings:



VIII. COUPLING BETWEEN EP FORMATION AND CALCIUM TRANSLOCATION

As stated in the previous section, the formation of the phosphorylated intermediate EP from E and ATP required the presence of Ca^{2+} outside the membrane; the reverse reaction, the formation of ATP from EP and ADP, required the presence of Ca^{2+} inside the membrane vesicles. Furthermore, it was suggested that the formation of EP was accompanied by a great change in the affinity of the enzyme for Ca^{2+} and Mg^{2+}. In this section, we describe more direct evidence for the coupling of the Ca^{2+} translocation with the elementary steps of the ATPase reaction.

A stoichiometric coupling of 2 mol of Ca^{2+} translocated for each mole of ATP hydrolyzed was found to exist during the active transport of Ca^{2+} (sect. VI*B*). However, analysis of the overall reaction does not provide the infor-

mation required to clarify the molecular mechanism of this coupling. In order to obtain more direct information on the mechanism of coupling between the calcium transport and the ATPase reaction, the time course of the translocation of calcium during a single cycle of ATP hydrolysis must be compared with the time course of the elementary steps in the ATPase reaction.

A thorough examination of the coupling between the translocation of calcium and the formation of EP was performed by Sumida and Tonomura (344). Selecting conditions, such as the exclusion of added Mg^{2+}, relatively low concentration of Ca^{2+} (20 μM), and low temperature (0°C), they examined the time courses of the formation and decomposition of EP and compared them with the time course of calcium transport. Under these conditions, EP was rapidly formed while the liberation of P_i was extremely slow. The slow rate of P_i liberation was due to the restraint of EP decomposition caused by the absence of added Mg^{2+} (sect. VII*D*). When the amounts of calcium uptake into the vesicle were measured at various times, by stopping the reaction by the addition of EGTA and a large amount of Mg^{2+}, the time course of calcium uptake exhibited a fast initial phase, corresponding to the time course of the EP formation, followed by a slower steady phase, corresponding to the time course of slow P_i liberation. When calcium uptake was terminated by the addition of EGTA and ADP, its time course exhibited only the slower steady phase, corresponding to the liberation of P_i. Based on the previously mentioned findings (sect. VII*C*, *D*) that EP decomposes into E and P_i on addition of EGTA and Mg^{2+}, whereas ATP is formed from EP and ADP on addition of EGTA and ADP, the observations of Sumida and Tonomura (344) can be interpreted in the manner summarized in the following equation:

$$\begin{array}{l} E + 2Ca^{o} + ATP \rightleftharpoons E_{ATP}^{Ca_2^{o}} \rightleftharpoons E_{P}^{Ca_2^{i}} \xrightarrow{Mg^{2+}} E + P_i + 2Ca^{i} \\ \quad\downarrow \qquad\qquad\qquad\qquad\qquad + ADP \\ EGTA \cdot Ca^{o} \end{array} \tag{4}$$

When EP was decomposed by addition of EGTA and Mg^{2+}, calcium translocated during the EP formation remained within the vesicle. In contrast, when EGTA and ADP were added under the same conditions, calcium previously translocated to the inside of the vesicle by the EP formation was released to the outside medium, due to the formation of EATP from EP and ADP. In fact, when a large amount of ADP was added immediately after EP formation was terminated by the addition of EGTA and Mg^{2+}, the vesicle released an amount of calcium almost equal to that taken up during EP formation. Furthermore, the amount of calcium released under these conditions decreased as the interval between the addition of EGTA and Mg^{2+} and the addition of ADP increased, and the time course of the decrease in the amount of calcium released by adding ADP corresponded closely to that of the decomposition of EP after the addition of EGTA and Mg^{2+}. Based on these findings, Sumida and Tonomura (344) concluded that EP formation

was coupled with the translocation of calcium from outside to inside the membrane, that EATP formation from EP and ADP was coupled with the translocation of calcium from inside to outside the membrane, and that EP formation induced a marked decrease in affinity of Ca^{2+} to the ATPase enzyme and Ca^{2+} remained within the vesicle when EP was decomposed into E + P_i.

Makinose (214) was the first to find that the reversible translocation of calcium across the membrane of the sarcoplasmic reticulum was closely correlated with EP formation. He observed that both the influx and efflux of calcium across the membrane were enhanced markedly when ADP and Ca^{2+} were added to vesicles that were partially preloaded with calcium in the presence of ITP and the precipitating anion P_i. Under these conditions, the ITP-ADP exchange that was previously shown to be coupled with the formation of EP was markedly activated. In contrast, the ITP-P_i exchange, as an index of the reversal of the calcium pump (see sect. x), was completely suppressed.

It should also be stressed that the formation of EP is accompanied by a great reduction in the affinity of the enzyme for Ca^{2+}, thus causing calcium to be released into the interior of the vesicle, as mentioned in section VII*D*. Recently, Ikemoto (127) examined the time courses of calcium binding and EP formation by a purified ATPase preparation that was capable of binding calcium but incapable of accumulating this cation. He found that the bound calcium was released from the enzyme on formation of EP, whereas calcium was reassociated with the enzyme when EP was decomposed into E and P_i. Further, Ikemoto (129) analyzed the transient-state kinetics of the formation of EP and calcium release by means of a rapid-mixing, acid-quenching method and by a stopped-flow technique employing a Ca^{2+} indicator dye, Arsenazo III. He found that although the EP formation started immediately after being mixed with ATP, the release of Ca^{2+} began only after a significant delay and so reached the maximal level at a considerably slower rate than did EP formation. Ikemoto (129) suggested that these observations were explained by sequential formation of two acid-stable phosphorylated intermediates having different affinities for Ca^{2+}. The first, having a higher Ca^{2+} affinity, was formed immediately after the addition of ATP. This high-affinity intermediate was then transformed to one with lower affinity, allowing the bound calcium to be released. Although these observations give more detailed insights into this process, the exact correlation at the molecular level between the phosphorylation and subsequent alteration in the affinity for cations remains to be determined.

IX. REGULATION OF CALCIUM TRANSPORT

The Ca^{2+}-dependent ATPase activity of sarcoplasmic reticulum can be regulated by several control systems. We describe three of these in the light of the reaction mechanism of Ca^{2+}-dependent ATPase that has been detailed

in the preceding two sections. In addition, we also describe a regulatory mechanism of the ATPase by cyclic AMP-dependent protein kinase, which has been found to exist in the cardiac sarcoplasmic reticulum.

A. Stimulation by ATP

The substrate ATP was found to accelerate the ATPase reaction. Yamamoto and Tonomura (412) and Kanazawa et al. (159) found that both V_{max} and apparent K_m of the steady-state ATPase at higher ATP concentrations were greater than those at the lower concentrations. Effects of temperature, pH, and treatment with *N*-ethylmaleimide on the rate of ATP hydrolysis of a given preparation were also identical at both low and high ATP concentration ranges (412). More recently, Dupont (56a) observed that PP_i and adenosine 5′-(α,β-methylene) triphosphate, which were hydrolyzed very little by the ATPase enzyme, induced remarkable activation of the ATPase activity. These findings suggested that ATP could act on the enzyme not only as a substrate but also as a regulator controlling its activity. Similar effects of ATP were also found in myosin ATPase (362, 369) and Na^+-K^+-dependent ATPase (365, 367).

A double-reciprocal plot of the concentration of the reaction intermediate (EP) at the steady state against the ATP concentration gave two straight lines, characteristics essentially identical to those of ATP hydrolysis (159). Thus, the ratio of the rate of P_i liberation (v) to the concentration of EP ([EP]) was virtually independent of ATP concentration (159). Direct measurement of the rate of EP decomposition indicated that high concentrations of ATP did not affect the decomposition of EP (159). These findings supported the view that the remarkable stimulation of the ATPase activity by high concentration of ATP resulted from the ATP-induced acceleration of formation of the intermediate. Actually, the double-reciprocal plot of the initial rate of formation of EP at high ATP concentrations exhibited a downward deviation from linearity (159), resembling those of the overall ATP hydrolysis and the amount of EP at the steady state. In contrast, other investigators suggested that the high ATP concentrations might influence the decomposition of EP (51, 88). However, by direct measurement of the rate of decomposition of EP by a quench-flow experiment, Martonosi et al. (239) confirmed our observation that high ATP concentrations had no effect on the rate of EP decomposition.

The formation of the Michaelis complex from the initial reactants (E + ATP $\rightleftharpoons$ E·ATP) is a very rapid process and was always in quasi-equilibrium (sect. VII). It is also evident that the rate of EP formation from its direct precursor was not accelerated by ATP, because the reverse reaction, i.e., the formation of ATP from EP and ADP, was not stimulated by ATP (159). Therefore, Kanazawa et al. (159) assumed the existence of two types of the Michaelis complex, $_1$EATP and $_2$EATP, and proposed that the rate of formation of the second complex from the first was accelerated by high concentrations of ATP (159), as shown in *equation 5*:

$$E + ATP \rightleftharpoons {}_1EATP \longrightarrow {}_2EATP \rightleftharpoons EP + ADP \qquad (5)$$

rapid equilibrium

accelerated by high ATP concentration

The scheme is essentially similar to the mechanism by which high concentrations of ATP were proposed to stimulate myosin ATPase (362, 369) and Na^+-K^+-dependent ATPase (365, 367).

B. Calcium-Induced Inhibition of EP Decomposition

The second possible regulatory process involves the Ca^{2+}-induced inhibition of EP decomposition. Yamada and Tonomura (408) found that Ca^{2+} induced a competitive inhibition of the Mg^{2+}-dependent decomposition of EP as part of the reaction sequence in which the affinities of the ATPase enzyme for Ca^{2+} and Mg^{2+} changed dramatically during the formation and decomposition of EP (see sect. VII). As the observed inhibition by Ca^{2+} presumably takes place at the inside of the membrane (see sect. VIII), accumulation of Ca^{2+} within the vesicle should lead to the decrease in the ATPase activity. Indeed, Weber (393, 394) showed that the Ca^{2+}-dependent hydrolysis of ATP by sarcoplasmic reticulum vesicles decreased during the initial phase of the reaction when there was considerable uptake of calcium into the vesicle. She suggested that such inhibition of the ATPase could be an important factor regulating the function of sarcoplasmic reticulum (393).

C. Initial Transition of ATPase Activity

A third possible regulatory process involves the large transition of v/[EP], i.e., the ratio of the rate of P_i liberation to the concentration of the phosphorylated intermediate, during the initial phase of the reaction. Kanazawa et al. (159) reported that the time course of P_i liberation during the reaction of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum consisted of a lag phase, a burst phase, and a steady phase, whereas the phosphorylated intermediate (EP) reached maximal levels immediately after the start of the reaction. Under these conditions, therefore, the value of v/[EP] exhibited a pronounced transition from a high value during the initial phase to a small one at the steady state (159, 410). Thus, there was a dramatic decrease in the value of v/[EP] to less than $^1/_{10}$ the initial value within a few seconds after the addition of ATP. The decrease in v/[EP] corresponded closely to the decrease in the first-order rate constant (k_d) of EP decomposition during the initial phase of the reaction (159, 410). Based on these findings, Kanazawa et al. (159) suggested that the burst phase of P_i liberation is caused by the transition of the k_d value. A more recent observation by Sumida et al. (342) indicated that the value of k_d at the initial phase is more than 7 times greater than that at the steady phase and that the transition of k_d takes place according to the following equation:

$$k_d = (k_{d,\text{initial}} - k_{d,\text{steady}}) \times \exp(-k_{tr} \cdot t) + k_{d,\text{steady}} \quad (6)$$

where $k_{d,\text{initial}}$ and $k_{d,\text{steady}}$ are the values of k_d at the initial and steady phases of the reaction, respectively, and k_{tr} is the rate constant for transition in the k_d value during the initial phase. Sumida et al. (342) reported that in a typical experiment the ratio of $k_{d,\text{initial}}$ to $k_{d,\text{steady}}$ was 7.3 and the value of k_{tr} was 0.5 s^{-1}. They also showed that the time courses of P_i liberation calculated from the observed rates of EP formation and the values of k_d obtained from the above equation were in good agreement with experimentally observed time courses of P_i liberation (342).

In contrast to the view that the initial P_i burst is derived from a transition in the rate constant of decomposition of EP, an alternative mechanism for explaining the initial transition in $v/[EP]$ was proposed recently by Froehlich and Taylor (88, 89), who assumed the existence of a TCA-labile phosphorylated intermediate E·P in addition to the TCA-stable EP. They suggested that the initial burst of P_i liberation is derived from the decomposition of the labile intermediate, E·P, which is considered to be in quasi-equilibrium with EP. The reaction mechanism proposed by Froehlich and Taylor (88, 89) may be useful in explaining the complex time course of the initial phase of EP formation and P_i liberation. However, the possibility that an appreciable amount of E·P, a hypothetical intermediate, exists in equilibrium with EP can be ruled out by three observations: *a*) the sum of the amounts of EP formed and P_i liberated is equal to the amount of decrease in ATP (159); *b*) during the reverse reaction of the ATPase (sect. x), the amount of ATP formed from EP and added ADP is equal to the amount of decrease in EP (406, 407); and *c*) although the amount of the initial burst of P_i liberation reaches 5 times that of EP, the maximal molar concentration of EP formed under optimal conditions does not exceed the molar concentration of the ATPase protein (159). However, it is not possible to rule out the existence of E·P as a labile intermediate when the backward reaction of the ATPase is carried out in the presence of large amounts of P_i (155, 156, 409).

It is of interest to assess the physiological significance of the initial transition in the value of k_d, in view of its potential importance in determining the initial reaction velocity of this system. Yamada et al. (410) suggested that the transition is caused by an ATP-induced cooperative change in the conformation of the vesicular structure of sarcoplasmic reticulum on the basis of the following observations: *a*) the transition in $v/[EP]$ is independent of the concentration of divalent cations inside the vesicles; *b*) the transition occurs even in the presence of a concentration of ATP too low to saturate the phosphorylation sites; and *c*) the transition is abolished when the membranes are treated with Triton X-100.

Tonomura (364) attempted to interpret the mechanism of the rapid transition by assuming that the ATPase could exist in either an active or inactive state, corresponding to the initial or the steady phase of the reaction. He suggested that the active state of the ATPase, such as that found during the initial phase where the k_d value is higher than that of the

inactive state at the steady phase, corresponds to the physiological state of the sarcoplasmic reticulum in the living muscle.

D. Regulation of Calcium Transport by Adenosine 3′:5′-Monophosphate and Protein Kinase

A regulatory mechanism important from the physiological and pharmacological points of view has been found to control calcium transport by the cardiac sarcoplasmic reticulum. In this section we first summarize briefly the basic properties of cardiac sarcoplasmic reticulum and review this new control mechanism of calcium transport, in which the phosphoester phosphorylation of a protein component of the cardiac membrane is assumed to play a key role in mediating the actions of hormones and drugs on heart muscle.

1. Cardiac sarcoplasmic reticulum

The steady-state characteristics of the ATP-supported calcium transport by sarcoplasmic reticulum from dog heart are generally similar to those of skeletal muscle sarcoplasmic reticulum, although the rate of calcium uptake by the former is lower than the latter (13, 23, 76, 105, 161, 162, 193, 340, 350, 399). A coupling ratio of 2 has been found to exist between the number of moles of calcium transported for each mole of ATP hydrolyzed (76, 350). An acid-stable phosphorylated intermediate, formed when cardiac sarcoplasmic reticulum is incubated with ATP, is labile in hydroxylamine (328, 341) and is associated with an ATPase protein with a molecular weight of 90,000–100,000 daltons (78, 193, 341). The amount of phosphoprotein intermediate formed has been found to be as high as 1.2–1.3 μmol/g of protein (282, 328), a value about one-fourth that of sarcoplasmic reticulum from rabbit fast-contracting skeletal muscle. The lower levels of the intermediate formed are in accord with the finding that the content of the 90,000- to 100,000-dalton ATPase in cardiac membranes is much lower: 35–40% (1, 341). Amino acid composition of the ATPase protein of dog cardiac muscle sarcoplasmic reticulum purified by gel filtration in the presence of SDS (350a) was quite similar to that of rabbit skeletal muscle sarcoplasmic reticulum (sect. IIIA). Thus, polar amino acids accounted for a rather large fraction (about 45%) of the total amino acids, and glutamic and aspartic acids accounted for about 22% of the total, outnumbering other polar amino acids (350a).

As in skeletal muscle sarcoplasmic reticulum, Ca^{2+} is required for the formation of the phosphorylated intermediate (EP), and Mg^{2+} is essential for its decomposition in the cardiac membranes (282, 328, 341). Kinetic analysis of ATPase of cardiac sarcoplasmic reticulum by Shigekawa et al. (328) indicates that the Ca^{2+} concentration required for half-maximal activation of the ATPase of cardiac muscle membranes (4.7 ± 0.2 μM) is 3–4 times higher than that for skeletal muscle membranes (1.3 ± 0.1 μM), whereas the turnover number of the Ca^{2+}-dependent ATPase of cardiac muscle membranes, calculated as the ratio of ATPase activity to the EP level, is not

significantly different from that of skeletal muscle membranes. Shigekawa et al. (328) concluded from these observations that a lower density of calcium-pumping sites and a lower affinity of these sites for Ca^{2+}, rather than a lower turnover rate, explained the relatively slow rate of calcium transport by cardiac sarcoplasmic reticulum.

2. *Phosphorylation of 22,000-dalton protein (phospholamban)*

Although the molecular mechanism of calcium transport by the cardiac sarcoplasmic reticulum is generally similar to that by skeletal muscle membranes, the cardiac membranes have recently been shown to possess a mechanism that is not seen in the membranes of fast-contracting skeletal muscle. Tada et al. (346, 350) and Kirchberger et al. (172, 174) reported that both calcium uptake and the Ca^{2+}-dependent ATPase of cardiac sarcoplasmic reticulum are stimulated by about threefold when adenosine 3′:5′-monophosphate (cyclic AMP)-dependent protein kinase catalyzes the phosphorylation of a 22,000-dalton protein (phospholamban) in the membranes of sarcoplasmic reticulum. This protein possessed stability characteristics of a phosphoester (172, 346), in which the phosphate was incorporated into serine (80%) and threonine (20%) (172).

A phosphoprotein phosphatase isolated from the myocardium that catalyzed dephosphorylation of the 22,000-dalton phosphoprotein (348) caused a significant decrease in the rate of calcium uptake (170), and a protein kinase modulator that inhibited cyclic AMP-dependent protein kinase activity abolished the cyclic AMP-dependent protein kinase-induced stimulation of calcium uptake and phosphorylation of the 22,000-dalton protein in cardiac sarcoplasmic reticulum (271, 351). Additional findings suggest that an intrinsic protein kinase associated with the membrane can catalyze the phosphorylation of this protein (191, 405).

The stimulation of calcium transport by cyclic AMP and protein kinase was seen both in the presence (350) and absence (403) of oxalate, and the stoichiometric coupling of 2 mol of calcium transported per mole of ATP hydrolyzed was maintained (350). The phosphorylation of the 22,000-dalton protein was accompanied by a significant reduction in the Ca^{2+} concentration required for half-maximal stimulation of the ATPase activity and the rate of calcium uptake, while the maximal rates for both were almost unchanged (350).

Based on these observations, Tada, Kirchberger, and Katz (164, 172, 173, 346, 347, 350) suggested that the formation and decomposition of the phosphoester phosphoprotein of 22,000 daltons catalyzed by cyclic AMP-dependent protein kinase and protein phosphatase, respectively, were capable of altering the rate of calcium transport by cardiac sarcoplasmic reticulum. They also suggested that the 22,000-dalton protein serves as a regulator of Ca^{2+}-dependent ATPase in the cardiac sarcoplasmic reticulum. This protein was tentatively named "phospholamban" ($\lambda\alpha\mu\beta\alpha\nu\epsilon\iota\nu$ = to receive), in view of its ability to receive phosphate from ATP (345, 346).

The proposal that the 22,000-dalton protein (phospholamban) exerts a critical control over the Ca^{2+}-dependent ATPase finds additional support in the apparent 1:1 stoichiometry existing between the amount of phospholamban phosphorylation [1–1.4 μmol/g of microsomal protein (169, 172)] and the amount of phosphoprotein intermediate of ATPase formed in this membrane (1.2–1.3 μmol/g of microsomal protein; sect. IX, *D1*). More recently, Tada et al. (350a) examined the effect of phospholamban phosphorylation on the kinetic properties of the ATPase enzyme in the absence of oxalate and found that the former phosphorylation did not alter the Ca^{2+}-dependence profile of the amount of the phosphoprotein intermediate of ATPase, but increased the rate of decomposition of the intermediate. However, it remains to be seen how these two phosphoproteins are related within the membrane of the cardiac sarcoplasmic reticulum.

3. *Physiological relevance of phospholamban phosphorylation*

If the findings on the effects of the cyclic AMP-protein kinase system on the calcium transport are relevant to the physiological behavior of the intact myocardium, they provide a biochemical explanation for the mediation by cyclic AMP of at least one of the principal mechanical effects of catecholamines on cardiac muscle: abbreviation of systole, because of the increased rate at which calcium is removed from troponin (164, 173, 345–349). It is also possible that an increase in both the rate of calcium release (164) and the amount of calcium stored in the sarcoplasmic reticulum could add to the amount of calcium available for delivery to the contractile proteins in subsequent contractions, thus promoting two other mechanical effects known to be produced by catecholamines: enhanced rate of tension rise and augmentation of contractility. Such a mechanism is consistent with the findings by Fabiato and Fabiato (77) that cyclic AMP increased both the rate and amount of calcium released from sarcoplasmic reticulum of a skinned cardiac muscle cell.

That phospholamban is functional in the membranes of other types of muscle was first suggested by Kirchberger and Tada (171), who reported that microsomes isolated from slow-contracting skeletal muscle (rabbit and cat soleus, and dog biceps femoris) contained a protein similar to phospholamban. Phosphorylation of the latter membranes by cyclic AMP-dependent protein kinase results in a significant increase in the rate of calcium uptake, whereas microsomes isolated from fast-contracting skeletal muscle (rabbit and cat tibialis anterior) did not form a 22,000-dalton phosphoprotein nor was their calcium uptake stimulated by protein kinase. Kirchberger and Tada (171) suggested that the observed effects of protein kinase on microsomes of slow-contracting muscle were related to the relaxation-promoting effect of catecholamines in this type of muscle (20), whereas the absence of a relaxation-promoting effect of catecholamines in fast-contracting muscle (20) was associated with the absence of a protein kinase substrate in the sarcoplasmic reticulum of this type of muscle.

Schwartz et al. (317) largely confirmed the findings of Tada, Kirchberger, and Katz on microsomes from cardiac and slow-contracting muscle. In disagreement with Kirchberger and Tada (171), however, Schwartz et al. (317) found that cyclic AMP-dependent protein kinase increased the rate of calcium uptake by microsomes from cat tibialis muscle, but without accompanying phosphorylation of the membrane. Furthermore, these authors reported that phosphorylase kinase was capable of both phosphorylating a 95,000-dalton protein and stimulating calcium uptake in microsomes from cardiac and skeletal muscles. The discrepancy between these two groups regarding the existence of this regulatory mechanism in different muscle types remained to be resolved. It also remains to be seen whether phosphorylase *b*, reported to exist within cardiac microsomes (74), has a role in regulating calcium transport.

The phosphorylation of phospholamban in sarcoplasmic reticulum might represent just one means of physiological regulation of contractility; other regulatory mechanisms such as phosphorylation of troponin (37, 73, 311, 335, 339) and myosin light chains (85, 272, 288) are also being considered.

X. COUPLING OF ATP SYNTHESIS WITH CALCIUM TRANSPORT

This section outlines the energetics of the backward reaction of the ATPase reaction that is coupled with the reversal of calcium transport. One of the most striking achievements in modern bioenergetics is the finding that ATP can be synthesized by a reverse reaction involving the ATPase protein of various transport systems, including the sarcoplasmic reticulum membrane. This achievement received wide attention from a bioenergetic point of view, since it lent support for the chemiosmotic hypothesis, originally proposed by Mitchell (254) to explain the molecular mechanism of ATP synthesis coupled with H^+ movements down a gradient in mitochondria and chloroplasts. Active calcium transport by the membranes of the sarcoplasmic reticulum was considered to be especially suitable to examine the adequacy of the chemiosmotic hypothesis, since both the reaction sequence and the mechanism of coupling between the ATPase and transport are well understood.

A. Synthesis of ATP in the Presence of Concentration Gradient for Ca^{2+}

The membrane of sarcoplasmic reticulum is impermeable to Ca^{2+} (sect. v, *D2*) so that when vesicles formed from this membrane are loaded with Ca^{2+}, either by active transport or by simple diffusion, subsequent reduction of the external Ca^{2+} concentration by the application of EGTA to the medium induces only very slow leakage of Ca^{2+} from the vesicle. Thus, the presence of a concentration gradient for Ca^{2+} across this membrane does not enhance its permeability to Ca^{2+} (41, 148, 406). However, when ADP, P_i, and Mg^{2+} are present when there is a gradient between internal and external

Ca^{2+}, efflux of Ca^{2+} from the vesicle is enhanced markedly (12). These findings led Barlogie et al. (12) to suggest that the Ca^{2+}-dependent ATPase reaction of sarcoplasmic reticulum could be reversed. Makinose (212) found that the ADP- and P_i-dependent efflux of Ca^{2+} from Ca^{2+}-loaded vesicles was accompanied by an ATP-P_i exchange reaction. De Meis and co-workers (47, 48, 50, 55) obtained essentially similar results, and Racker (299, 304) showed that reconstituted sarcoplasmic reticulum membranes exhibited efflux of Ca^{2+} under similar conditions. Makinose et al. (212, 213, 216) and Panet and Selinger (281) observed that the addition of ADP, P_i, and EGTA to Ca^{2+}-loaded vesicles resulted in the formation of 1 mol of ATP during the release of each 2 mol of Ca^{2+}. These results indicate that the membranes of sarcoplasmic reticulum are able to synthesize ATP by expending the osmotic energy produced by movement of Ca^{2+} down the concentration gradient for Ca^{2+} across the membrane.

In view of the potential importance of the ATP synthesis in the chemiosmotic coupling of calcium transport, Yamada et al. (406, 407, 409) and Makinose (213) extensively investigated the kinetic properties of ATP synthesis from ADP and P_i in light of the reaction mechanism of the ATPase, the details of which are documented in section VII. In order to obtain Ca^{2+}-loaded vesicles, Makinose (213) utilized acetylphosphate to energize the active transport of calcium, whereas Yamada et al. (406, 407, 409) incubated the vesicles with a large amount of Ca^{2+} in the absence of the energy donor for several hours at 0°C in order to raise the electrochemical activity of Ca^{2+} inside the vesicle toward that of the medium. The concentration of Ca^{2+} in the medium was subsequently reduced by the addition of EGTA at a large excess over $CaCl_2$, thus producing a concentration gradient of Ca^{2+} across the membrane. Yamada et al. (406, 407) and Makinose (213) reported almost simultaneously that the addition of Mg^{2+} and $^{32}P_i$ under these conditions caused ^{32}P to become incorporated into the ATPase protein. The same reaction occurred even after the membranes were solubilized, i.e., in the absence of the concentration gradient of Ca^{2+} (see below). Since the latter reaction is accompanied by a great increase in entropy, the reaction temperature was lowered to minimize the incorporation of P_i in the absence of concentration gradient of Ca^{2+} (406). Yamada et al. (406, 407, 409) found that the amount of P incorporated ([EP]) in the steady state depended on the concentration gradient of Ca^{2+} across the membrane and the concentrations of Mg^{2+} and P_i in the medium according to the following equation:

$$\frac{[EP]}{\epsilon} = \frac{1}{1 + \phi\left(\frac{\{Ca^{o}\}}{\{Ca^{i}\}}\right)^{2}\frac{1}{[Mg][P_i]}}$$

where ϵ represents the total concentration of the phosphorylation sites and $\{Ca^{i}\}$ and $\{Ca^{o}\}$ represent the electrochemical activities of Ca^{2+} inside and outside the membrane, respectively. As mentioned above, the value of $\{Ca^{i}\}$ was equal to that of the medium in which loading of the vesicles with Ca^{2+}

had been achieved, whereas the value of $\{Ca^{o}\}$ was estimated from the binding constant between calcium and EGTA. This equation indicates that the amount of EP depends on the square of the ratio $\{Ca^{o}\}/\{Ca^{i}\}$, suggesting that 2 mol of Ca^{2+} react with each mole of the phosphorylation site of the ATPase protein. Furthermore, as reported by Kanazawa and Boyer (156), a small amount of the extravesicular Ca^{2+} markedly inhibits the formation of EP from P_i by competing with Mg^{2+}. Under optimal conditions, all the sites could be phosphorylated. The free-energy change during P_i incorporation due to chemiosmotic coupling was calculated to be about 12 kcal/mol at pH 7.0 and 20°C. The chemical characteristics of the phosphoprotein formed in this reaction were indistinguishable from that formed during the forward reaction of ATPase.

Addition of ADP after the formation of EP from E and P_i results in the rapid synthesis of ATP. Under these conditions, 1 mol of ATP is formed for each 2 mol of Ca^{2+} existing within the vesicle. Based on these observations, Yamada et al. (406) concluded that the ATP synthesis that was energized by the concentration gradient for Ca^{2+} across the membrane represents the reverse reaction of the Ca^{2+}-dependent ATPase. In this reverse reaction, therefore, the vectorial properties of the enzyme for Ca^{2+}, P_i, ADP, and ATP are expected to be maintained. This prediction is supported by the finding by Yamada et al. (406) that the presence of ATP outside the membrane inhibited the formation of EP from E and P_i, and the observations of de Meis and M. G. C. Carvalho (49), who demonstrated that intravesicular P_i (incorporated as a precipitating anion for Ca^{2+}) cannot serve as a substrate for EP formation or ATP synthesis through the reverse reaction. The latter authors (49) also reported that the synthesized ATP was released to the exterior of the vesicle.

B. Synthesis of ATP in the Absence of Concentration Gradient for Ca^{2+}

Kanazawa and Boyer (154–156) found that the Ca^{2+}-dependent ATPase could be phosphorylated by P_i, even when the membranes of sarcoplasmic reticulum were solubilized with Triton X-100 (155) or made leaky to Ca^{2+} with alkali (154, 156). Further evidence that a concentration gradient of Ca^{2+} is not always required to form EP from E and P_i was obtained in ether-treated sarcoplasmic reticulum membranes (54, 241). Kanazawa (155) also showed that EP formed from E and P_i in the absence of concentration gradient of Ca^{2+} is an acylphosphate, like EP formed by the reaction of E with ATP. The energy source for formation of EP by the backward reaction is documented below (sect. x*C*). From a kinetic analysis of the dependence of the rate of EP formation on the concentration of P_i, Kanazawa (155) suggested that EP was formed via the enzyme-phosphate complex $E \cdot P_i$. Knowles and Racker (176) demonstrated that the purified ATPase preparation was capable of forming EP by the reaction with P_i. Like the formation of EP from E and P_i in the presence of a concentration gradient for Ca^{2+}

(sect. x*A*), the formation of EP from E and P_i in the absence of this concentration gradient requires the presence of a high concentration of Mg^{2+} and is inhibited competitively by a small amount of Ca^{2+} in the external medium (154, 155, 176). The observation that this inhibition by Ca^{2+} has a Hill coefficient of 2 (155, 156) is in accord with the observed stoichiometry of 2 mol of Ca^{2+} reacting with each mole of ATP during the forward reaction.

Knowles and Racker (176) observed ATP synthesis from P_i and ADP in the absence of a concentration gradient for Ca^{2+}. After incubation of the purified ATPase with P_i in the presence of EGTA to promote EP formation, a large amount of Ca^{2+} was added to terminate the formation of EP. Subsequent additions of ADP caused ATP to form in an amount equal to the amount of EP that had existed previously. Knowles and Racker also reported that the newly synthesized ATP was not bound to the enzyme (176).

An ATP-P_i exchange reaction was examined by de Meis and co-workers (28, 48, 50, 55) and Racker (299) as an index of the reversible reaction of Ca^{2+}-dependent ATPase in a leaky membrane system where no concentration gradient for Ca^{2+} could be present. An exchange reaction found in the absence of the concentration gradient was enhanced remarkably when the concentration of Ca^{2+} was increased from 0.1 to 10 mM (48, 50, 55, 299). These findings are apparently inconsistent with the observation that a small amount of Ca^{2+} suffices to inhibit the EP formation from E and P_i in the absence of a concentration gradient for Ca^{2+}. The discrepancy remains to be solved.

Vale et al. (375) indicated that use of calcium ionophore X537A allows two states in which calcium exists within the sarcoplasmic reticulum vesicles to be distinguished. They suggest that the first state, i.e., calcium that was released by X537A, represents intravesicular Ca^{2+} and the remaining calcium exists as membrane-bound calcium. Vale et al. (376) demonstrated that when ADP, P_i, and EGTA are added to the Ca^{2+}-loaded vesicles at 25°C a significant amount of ATP is formed by release of the membrane-bound calcium as well as by release of the total calcium. The ATP synthesis due to the release of membrane-bound calcium in this experiment presumably corresponded to the synthesis of ATP in the absence of a concentration gradient for Ca^{2+}.

Thus, three types of ATP synthesis from ADP and P_i have been found to be catalyzed by the sarcoplasmic reticulum under three different conditions: in the presence of a concentration gradient for Ca^{2+} (intact vesicles) (213, 216, 281, 406, 407, 409), in its absence (solubilized system) when Ca^{2+} is removed from the vesicles (155, 176), and when Ca^{2+} is raised to high concentrations (48, 55, 299, 376). It is not understood, however, how the basic mechanisms of these different types of ATP synthesis are related to each other.

C. Thermodynamic Analysis of EP Formation

From a thermodynamic examination of the formation of EP from E and P_i in the solubilized system, Kanazawa (155) reported that the formation of

EP from $E \cdot P_i$ complex was accompanied by a remarkably large increase in entropy. Based on the analysis of the temperature dependence of the equilibrium between EP and $E \cdot P_i$ ($E \cdot P_i \rightleftharpoons EP$), Kanazawa (155) calculated the values of the standard free energy, enthalpy, and entropy of the equilibrium as +0.4 kcal/mol, +16 kcal/mol, and +50 eu, respectively. These findings led Kanazawa (155) to suggest that EP was thermodynamically stabilized by the increase in entropy on its formation from the enzyme-phosphate complex. Thus, the enzyme was phosphorylated by P_i to form acylphosphate without any supply of osmotic energy or any other form of exogenous high energy. Further, Kanazawa (155) suggested that when the transport process was driven in the forward direction by ATP the entropy of the transport system greatly increased in some earlier step and returned to the initial level in the step of the decomposition of EP. It was not clear at this stage the elementary step of the transport process with which the great change in entropy and enthalpy should be associated. Such a bioenergetically important question may be answered when the thermodynamic properties of the reverse reaction in the presence and absence of the concentration gradient of Ca^{2+} are clarified thoroughly.

XI. LIPID-PROTEIN INTERACTIONS IN THE MEMBRANE AND MOLECULAR STRUCTURE OF ADENOSINETRIPHOSPHATASE

In this section we describe the physicochemical properties of the major components of the sarcoplasmic reticulum and the interactions that are essential for their functional manifestation as ATPase activity and calcium transport. Emphasis is placed on the dynamic properties of the lipid-ATPase interactions and the submolecular distribution of the physiological activities in the ATPase protein. More exact knowledge of these questions should provide a basis for the construction of a molecular model that can explain the mechanism of energy transduction during calcium transport by the sarcoplasmic reticulum.

A. Interactions Between Lipid and ATPase

1. Role of boundary phospholipids

As mentioned in sections II*D* and III*A*, the ATPase of the sarcoplasmic reticulum is an amphipathic single polypeptide with a molecular weight of about 100,000, whose hydrophobic region is in direct contact with the membrane lipid. In the intact sarcoplasmic reticulum, phospholipids make up a large part (about 80%) of total lipid, the remainder being neutral lipid (mainly cholesterol) (sect. II*B*). Although bilayer lipid forms the basic framework of the membrane, phospholipids tightly bound to the ATPase protein are also known to exist.

In 1973 Jost et al. (151, 152) interpreted studies with lipid spin label to indicate that a single layer of the protein-bound phospholipids is essential in

maintaining the activity of cytochrome oxidase isolated from beef heart mitochondria. Jost et al. (151, 152) suggested that an annular layer of phospholipids in direct contact with the hydrophobic region of the protein provided a boundary between the fluid bilayer region and the membrane protein, thus helping maintain a protein structure that is essential for the manifestation of the activity. Such a single shell of the phospholipid bilayer was referred to as an annulus of boundary phospholipids.

Existence of a boundary lipid in the membrane of sarcoplasmic reticulum was first indicated by Warren and co-workers (385, 386), who found that about 30 mol of lipid were tightly bound to 1 mol of ATPase, forming a boundary annulus surrounding the protein. When the purified ATPase of sarcoplasmic reticulum having more than 30 mol of lipid/mol was delipidated by increasing amounts of deoxycholate, full ATPase activity was maintained only at a molar ratio above 30. At lower ratios there was an irreversible loss of activity that reached a negligible level at about 15 lipid molecules for each mole of the ATPase (386). Employing dinitrophenylated dipalmitoyl phosphatidylethanolamine, Hardwicke (103) attempted to determine the number of phospholipid-binding sites in delipidated, deoxycholate-free ATPase protein (104). The findings suggested that the total number of molecules of the phospholipid capable of binding to the lipid-free ATPase protein was about 15.

The boundary lipid could be reversibly titrated by the synthetic phospholipids such as dioleyllecithin (DOL, 18:1; 18:1), dipalmitoyllecithin (DPL, 16:0; 16:0), and dimyristoyllecithin (DML, 14:0; 14:0). This was achieved by dissolving the purified ATPase in a mixture containing deoxycholate and any one or combination of a large excess of the synthetic phospholipids and then centrifuging the protein into a detergent-free sucrose gradient (386). More than 99% of the endogenous lipids could be successfully replaced by the added lipids. Complete replacement of the annular lipid by DOL, DPL, or DML restored the ATPase activity (384, 386, 387) and the temperature profiles of the ATPase activity of such enzyme-lipid complex were determined to a large extent by the temperature dependence of the phase transition of individual phospholipids substituted (see below).

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 the Ca^{2+}-dependent ATPase activity (384). However, the presence of a high concentration of deoxycholate allowed cholesterol to replace the annular lipid. This replacement caused a complete and reversible inactivation of the ATPase activity; complete loss of the activity was observed at 15 mol of cholesterol replaced per mol of ATPase (384). These findings suggest that about 30 lipid molecules interact directly with the hydrophobic part of each ATPase molecule to support full enzymic activity. The reversible inactivation when the ATPase protein was complexed with saturated lecithins and cholesterol demonstrates that a highly rigid interaction is able to inhibit completely ATPase activity. Another possible explanation for inhibition by cholesterol is that it does not meet the

requirement of the protein for specific chemical structures in the annulus. This model of the ATPase-lipid complex is essentially similar to that of cytochrome oxidase, mentioned above.

More recently, Hesketh et al. (117) studied the temperature profile of the ATPase activity and lipid spin label in the ATPase preparation whose lipid was replaced to various extents by 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 requirement (35 DPL molecules per ATPase), the complexes showed very similar temperature 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. The breaks in the Arrhenius plots of the ATPase activity, occurring at 27–32°C and at about 38°C, indicate some cooperative structural properties of the interaction of the annular DPL molecules with the ATPase. These findings led Hesketh et al. (117) to suggest that the annular phospholipids, by a tight binding to the enzyme, exhibit a conformational property much altered from their inherent nature and that such 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.

2. *Effect of phospholipids in formation and decomposition of EP*

Martonosi was the first to study extensively the physiological significance of phospholipids in sarcoplasmic reticulum (222, 224, 225). He and his co-workers demonstrated that treatment of sarcoplasmic reticulum with phospholipase C inhibited ATPase activity and calcium transport, the extent of inhibition being proportional to the extent of hydrolysis of membrane lecithin (225). Both activities were reactivated by the addition of lecithin. Martonosi and co-workers (233, 234) and Meissner and Fleischer (249) 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 findings suggested that the decomposition of the phosphorylated intermediate of ATPase required the existence of phospholipids. In contrast, Fiehn and Hasselbach (80) reported that delipidation by phospholipase A resulted in decreased phosphoenzyme formation. The conclusions of the former investigators were later supported by the observations of M. Nakamura and Ohnishi (266), who reported that delipidation by phospholipase A caused proportional losses of both ATPase activity and phospholipid content with no effect on phosphoenzyme levels. Hardwicke and Green (104) and Knowles et al. (175) 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

obtained from various sources (175). H. Nakamura et al. (264) and Hidalgo et al. (118) demonstrated that in an ATPase preparation whose phospholipids were substituted by synthetic phospholipids (118, 264), the decomposition of the phosphoprotein intermediate was influenced significantly by the nature and fluid state of protein-bound annular phospholipids, whereas its formation was not affected by these factors. The conclusion of Nakamura and Ohnishi (266) that the loss of the ATPase activity was related to the loss of the fluid bilayer lipid is not apparently in accord with that of Hesketh et al. (117), who showed that the annular lipid had a predominant effect on ATPase activity. Although this discrepancy might reflect differences in the sources of lipid used in the experiments, the latter authors failed to determine whether the decreased ATPase activity was due to a decrease in the formation or in the decomposition of the phosphorylated intermediate.

3. Fluidity of bilayer lipid

Many attempts were made to define the properties of fluid bilayer lipid of sarcoplasmic reticulum by determining the fluidity of bulk phospholipids (38, 68, 118, 122a, 143, 240, 242, 256, 266, 316) and relating these findings to the ATPase and calcium transport activities (38, 118, 143, 208, 209, 259, 264). Inesi et al. (143) were the first to relate the temperature dependence of the 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. The observed temperature profile was suggested by Inesi et al. (143) to represent a dual function of the phase transition of bilayer phospholipids and the temperature dependence of the ATPase activity. However, the latter activity has recently been found to be determined largely by the phase transition of the protein-bound annular phospholipids, which was much different from that of bulk phospholipids in the fluid bilayer (117), as stated above (sect. XI, *A1*).

B. Proteolytic Subfragmentation of ATPase

It is difficult to construct a molecular model that can substantially interpret the mechanism of calcium transport, in the absence of more accurate knowledge of the submolecular structure of the ATPase protein and the intramolecular distribution of the key functional features of the calcium transport ATPase. High-affinity calcium-binding sites (sect. V*A*) and a phosphorylation site or an ATP-binding site (sect. VI*E* and VII) have been shown to exist within the ATPase molecule of 100,000 daltons. The hydrophilic region of the ATPase is exposed to the exterior of the membrane, while its hydrophobic region interacts directly with boundary phospholipids (sect. II*D* and XI*A*). Proteolytic digestion of sarcoplasmic reticulum vesicles under various conditions has proved to be one of the most effective means by

which the intramolecular assignment of such key functional properties can be made.

Brief exposure of sarcoplasmic reticulum to trypsin in the presence of 1 M sucrose causes the ATPase protein to be degraded into two fragments with molecular weights of 52,000–60,000 and 45,000–55,000 (144, 195, 253, 336, 338, 359), as determined by electrophoresis on SDS-polyacrylamide gels (Table 2). The larger subfragment (fragment I) contains the phosphorylation site and probably the calcium-binding sites (see below). Both this and the smaller subfragment (fragment II) were separated to near homogeneity in the presence of various detergents by the use of column chromatography (309, 338, 360) or electrophoresis (360). Hydrophobicity of fragment II was only slightly higher than that of fragment I, as seen from the electrophoretic mobility in the presence of a limited amount of SDS on the cellulose acetate slabs (360), where the detergent presumably bound preferentially to fragment II. This interpretation is in accord with the finding that fragment II contains a slightly higher proportion of nonpolar amino acids than fragment I (338, 360). Thus, fragment I possessed about equal amounts of polar and nonpolar amino acids, while fragment II contained about 60% nonpolar and 40% polar amino acids (338).

Further proteolysis of fragment I yielded subfragments of 30,000–33,000 daltons and 20,000–24,000 daltons (338, 360), which are referred to as fragments Ia and Ib, respectively. When the active site of ATP hydrolysis was labeled with [γ-^{32}P]ATP (338, 359), or the thiol group that was protected by ATP (114, 279, 280; see sect. VI*F*) was labeled with *N*-ethyl-[2-^{3}H]maleimide (338), and the protein was subjected to fragmentation by trypsin, both labels were recovered in fragments I and Ia, indicating that these contain the active site of ATPase. There was antigenic cross-reactivity

TABLE 2. *Molecular weights of sarcoplasmic reticulum ATPase and its tryptic subfragments*

ATPase	Primary Degradation		Secondary Degradation		Reference
	Fragment I	Fragment II	Fragment Ia	Fragment Ib	
115,000	60,000	55,000	33,000	24,000	Thorley-Lawson and Green (359, 360)
102,000	55,000	45,000	30,000	20,000	Stewart, MacLennan, and co-workers (205, 336, 338)
100,000	60,000	49,000	32,000	21,000	Yamamoto and Tonomura (414a)
106,000	57,000	46,000			Inesi and Scales (144)
100,000	52,000	49,000			Louis et al. (195, 196)
120,000*	65,000*	56,000*			Yu et al. (419)
115,000 or 119,000†	56,000†	59,000†			Rizzolo et al. (309) and le Maire et al. (192a)

Values were determined by electrophoresis on SDS-polyacrylamide gels in all reports except those by Rizzolo et al. and le Maire et al. * ATPase protein labeled with diazotized diiodosulfanilic acid was subjected to subfragmentation. † Determined by sedimentation equilibrium measurements in SDS (309) or in deoxycholate (192a).

between fragments I and Ib, indicating that fragment Ib also originated from fragment I (338). Both fragments I and Ib were active in Ca^{2+}-dependent ionophore assays (see sect. XI*C*), suggesting that fragment Ib represented the substructure containing the calcium-binding sites of the parent molecule.

Stewart et al. (338) reported that none of the antibodies against the above-mentioned four fragments affected ATPase activity or calcium transport, whereas Sumida and Sasaki (343) found a significant inhibitory effect of anti-ATPase antibodies on calcium transport (see sect. XII*D*). The use of antibodies against each fragment indicated that fragment I is in large part exposed at the surface of sarcoplasmic reticulum vesicles, whereas fragments Ib and II are only poorly exposed (200, 338). These observations led MacLennan and co-workers (200, 338) to propose that the active site of the ATPase in fragment I is externally located, whereas the site with ionophore activity (fragment Ib) is only partially exposed and fragment II is largely buried within the membrane.

More recently, MacLennan et al. (203) demonstrated that fragments I and II, dissociated by brief exposure (less than 20 min) to solutions containing SDS, were capable of carrying out calcium transport when incorporated into phospholipid vesicles under the conditions for reconstitution described by Racker et al. (304).

C. Ionophore Activity

The existence of a Ca^{2+} ionophore activity within the ATPase protein is another interesting feature of this cation transport system. Substances that possess an ionophore activity may fall into two categories. An example of the first type is valinomycin, which acts as an intramembranous carrier for cations; gramicidin exemplified the other type, which makes a pore that allows cations to pass through biological membranes (276). Recently, Na^+ and Ca^{2+} ionophore activities were suggested to be present in large polypeptides isolated from the Na^+-K^+-dependent and Ca^{2+}-dependent ATPases. Shamoo and Albers (322, 323) were the first to indicate the possibility that Na^+-K^+-dependent ATPase from the electric eel could serve as a Na^+-ionophore. These investigators found that the addition of purified Na^+-K^+-dependent ATPase or its tryptic subfragments to a double-sector cell divided by an artificial bimolecular lipid membrane increased the Na^+ conductance across the membrane. Similar experiments were performed for the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum by Shamoo and MacLennan (324), who showed that when the purified ATPase solubilized by succinylation was added to the artificial membrane there was an increase in Ca^{2+} conductance. The increase became more pronounced when the ATPase protein was modified so as to be incorporated into the artificial membrane. The divalent-cation dependence of increase in conductance exhibited the selectivity: $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+}$ (324). Although these investigations suggest that the purified ATPase possesses a substructure containing calcium ionophore, it still remains to be seen whether the observed increase in conductance is too low to reflect the change due to

incorporation of the ATPase protein and its subfragments (fragments I and Ib) into bilayer membranes in their experimental system. According to their conclusion, however, we assume in this review that the 20,000-dalton fragment of the ATPase protein represents the calcium-binding moiety of the enzyme.

Of the four subfragments of the ATPase (sect. XI*B*), fragments I and Ib exhibited the Ca^{2+} ionophore activity (326). From the observation that fragment Ib possessing Ca^{2+} ionophore activity was only poorly exposed at the exterior of the membrane (sect. XI*B*), Stewart et al. (338) and Shamoo et al. (326) speculated that this part of the ATPase polypeptide was located at the mouth of the hydrophobic fragment II and served as the site at which the entry of Ca^{2+} was controlled.

The Ca^{2+} ionophore activity in the ATPase protein might explain the findings that the passive Ca^{2+} permeability of the intact sarcoplasmic reticulum is 10^4–10^6 times greater than that of liposomes (148). However, in the membrane reconstituted from the ATPase and lipid, an increase in passive Ca^{2+} permeability was also accompanied by increases in permeability to other inorganic agents, such as sucrose, Na^+, and choline (148; see sect. V, *D2*). On the basis of these observations, Jilka et al. (148) suggested that the permeability change arose from a nonspecific reordering of the structure of the lipid phase in the environment of the protein rather than from a carrier-mediated process.

The possibility that part of the ATPase possesses a Ca^{2+} ionophore activity may allow a more realistic model to be constructed in which a specific region of the ATPase polypeptide serves as either an intramolecular pocket or an intermolecular channel for Ca^{2+}. However, even if the 20,000-dalton ionophore protein constitutes the calcium-binding moiety of the ATPase, it remains unclear whether this moiety mediates the movement of Ca^{2+} from one side of the membrane to the other or whether it acts as a "gate" near the external surface.

D. Monomer or Oligomer?

It is of interest to consider whether the Ca^{2+}-dependent ATPase forms a monomer or an oligomer within the membrane to attain full activation of the calcium transport.

Using the Na^+-K^+-dependent ATPase of various membranes, many investigators attempted to determine the size of the functioning molecules by radiation inactivation or by use of a cross-linking agent. Kepner and Macey (165) and Nakao et al. (267) were the first to employ the radiation inactivation technique to estimate the functional molecular weight of the Na^+-K^+-dependent ATPase. The earlier studies had indicated relatively large values [1,000,000 (165) and 500,000 (267) daltons], but reestimation by Kepner and Macey (166) in studies performed in vacuo indicated that values ranged from 190,000 to 300,000 daltons. Kyte (184) obtained a molecular weight similar to the latter values for a product of the reaction catalyzed by a cross-linking agent (cupric phenanthroline). These observations indicate

the possible formation of a dimer or an oligomer, since the molecular weight of the Na^+-K^+-dependent ATPase monomer was reported to be about 100,000 (120, 150, 182, 189, 268). These findings are consistent with the recent observation by Jørgensen (150a) that only one of the two 100,000-dalton polypeptides is capable of undergoing phosphorylation. In the experiment by Kepner and Macey (166), however, the radiation inactivation of the Na^+-K^+-dependent ATPase was determined without determining the active transport of Na^+ and K^+ across the membrane, so that the observed value might not represent the true size of the functional molecules. In the experiment of Kyte (184) in which cross-linking was effected by a cupric agent, it is unclear whether the observed covalent dimer of the protein resulted from dimerization during random aggregation or from a specific dimerization between subunits of the functional transport ATPase. Therefore, it is premature to conclude from these experiments that the transport of Na^+ and K^+ across the membrane requires the formation of an oligomer of the transport enzyme. It appears likely that, for active Na^+ and K^+ transport in which the dimeric theory is often favored, there is little justification for the contention that dimer formation is an essential step. It thus remains to be seen whether the ATPase molecules exhibit movements of a condensation type during the process of cation transport.

In studies of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum, Vegh et al. (380) obtained a molecular weight of 190,000 by radiation inactivation of this enzyme and Murphy (257) obtained a molecular weight of 400,000 for a product of the reaction catalyzed by cupric phenanthroline. Although these results suggest the formation of a dimer or a tetramer, respectively, these values cannot be accepted as reflecting the size of the functional molecules for the reasons mentioned above. Employing sedimentation determination in nonionic detergent, le Maire et al. (192) found that the solubilized Ca^{2+}-dependent ATPase of sarcoplasmic reticulum exhibited a minimal molecular weight of about 400,000. However, it is not clear whether this represents the size of the functional molecules within the intact membrane. From thermodynamic arguments, Singer (330) proposed a molecular model in which the transport proteins existed as aggregates of two or more polypeptide chains (see sect. IV, *B2*). Similar arguments were presented by Dutton et al. (59), who assessed the applicability of this model in interpreting the molecular mechanism of calcium transport by sarcoplasmic reticulum, although no direct evidence to support this contention was presented (see sect. XII*E*). Therefore, it is premature to draw firm conclusions regarding the size of the functioning molecules from these observations.

More recently, Vanderkooi et al. (376a) analyzed the interaction between ATPase molecules in reconstituted ATPase vesicles by measuring the efficiency of energy transfer between two populations of ATPase molecules, one labeled with *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine serving as energy donor and the other with iodoacetamidofluorescein serving as energy acceptor. They found that the efficiency of energy transfer is not affected by dilution of the lipid phase of the reconstituted vesicles with egg

lecithin, suggesting that ATPase molecules exist as an oligomer. However, it is still unclear whether the observed interaction represents physiological states of the molecules.

XII. MOLECULAR MODEL OF ACTIVE CALCIUM TRANSPORT

A number of attempts have been made to explain the mechanism of active cation transport across the sarcoplasmic reticulum by assuming that the membrane ATPase serves as an energy transducer as well as a translocator of cations (sect. IV). The Ca^{2+}-dependent ATPase of sarcoplasmic reticulum assumes this essential role in the active transport of Ca^{2+} across this membrane (sect. III). In order to gain an understanding of the energy transduction by this system, the elementary steps in the reaction sequence of the ATPase were correlated with those of calcium transport (sect. VII, VIII, and X). The ATPase protein of sarcoplasmic reticulum exhibited unique structural features (sect. II and XI) and essentiality of the lipid interactions with this protein was documented (sect. XI). On the basis of these observations, we attempt in this section to assess what type of molecular model could substantially explain the functional changes in the ATPase that are coupled with calcium transport. Such a working hypothesis would in turn enable us to better understand the molecular mode of energy transduction in this and the other cation transport systems.

A. Adequacy and Limits of Shaw's Model

Kinetic analysis of the Ca^{2+}-dependent ATPase revealed a reaction sequence in which the ATPase enzyme forms an enzyme-substrate-calcium complex at the outer surface of the membrane, followed by formation of a phosphorylated intermediate that is coincident with the translocation of calcium from outside to inside the membrane (sect. VII and VIII). The phosphorylation of the enzyme is also associated with a dramatic change in the affinity for Ca^{2+} and Mg^{2+}, so that Mg^{2+}, having increased affinity for the phosphorylated intermediate, accelerates its decomposition (sect. VII). In the circulatory-carrier model of Shaw (see sect. IV*A*), it was assumed that the cation-binding site is translocated across the membrane, and this process is associated with a large change in the affinity of the binding site for cations. In this regard, therefore, this model may be adequate to explain the calcium transport by sarcoplasmic reticulum. However, this model is still incomplete, and additional evidence for its validity is needed. Thus, it should be known what kind of molecular motion of the ATPase protein is associated with the translocation of the calcium-binding site from outside to inside the membrane. The structural basis for the profound change in the affinities of the enzyme for cations should also be understood clearly.

B. Studies with Conformational Probes

Employing conformational probes such as spin labels and fluorescent dyes, many investigators attempted to detect a conformational change

associated with the transport of calcium across the sarcoplasmic reticulum membrane.

Landgraf and Inesi (141, 188) examined the electron paramagnetic resonance (EPR) spectrum of sarcoplasmic reticulum labeled with nitroxide-iodoacetate, and found that the spectrum showed two components, corresponding to a "weakly" immobilized and a "strongly" immobilized signal. Addition of ATP induced a change in the spectrum that indicated mobilization of a "strongly" immobilized component. They suggested that this alteration represented an ATP-induced conformational alteration in the protein structure. However, the observed change required high concentrations of ATP and was induced even in the absence of Ca^{2+} and Mg^{2+}. It therefore is possible that the change in the EPR spectrum represented an alteration produced by the ATP binding to the regulatory site (see sect. IX*A*), rather than that associated with the active site of the ATPase. H. Nakamura et al. (263) found that an ATP-dependent alteration in the EPR spectrum of sarcoplasmic reticulum labeled with nitroxide-maleimide required the presence of Ca^{2+} and Mg^{2+}, suggesting that a conformational change was associated with intermediary step(s) of the ATP hydrolysis. However, this change was not observed in the intact vesicle, but only after prolonged incubation of the vesicles with the spin label at alkaline pH in the presence of EGTA, a process that increased the leakiness of the membrane (see sect. V, *D2*). Therefore, the possibility remains that the observed alteration represents a conformational change that is not directly related to active calcium transport. More recently, Coan and Inesi (33, 34) reexamined the intriguing alteration in the EPR spectrum of sarcoplasmic reticulum labeled with nitroxide-iodoacetate (188). They found that the spectral change indicative of the mobilization of a "strongly" immobilized component was induced only in the presence of ATP, Ca^{2+}, and Mg^{2+} (34), as previously noted by Nakamura et al. (263). Although they suggested that ATP hydrolysis was not required for this alteration (34), an identical effect was obtained with acetylphosphate (33), which functions as a substrate for calcium transport (see sect. VI*C*). As described in section IX*A*, ATP could act on the ATPase not only as a substrate but also as a regulator controlling its activity, whereas acetylphosphate could not act as a regulator for the enzymatic activity. These observations led Coan and Inesi (33, 34) to suggest that a profound alteration in the structure of the ATPase was accompanied by the binding of substrates to the catalytic, rather than to the regulatory site. There is other indirect evidence supporting the close association between the formation of phosphorylated intermediate and change in the mobility of the enzyme-bound spin label (29, 283).

Employing anilinonaphthalene sulfonate (ANS), a fluorescent dye generally used as a conformational probe to study the polarity of the local environment, Vanderkooi and Martonosi (377–379) and Augustin and Hasselbach (9) attempted to determine a structural change associated with calcium transport by the membranes of sarcoplasmic reticulum. Vanderkooi and Martonosi (379) found that the extent of enhancement of the fluorescence intensity of ANS depended on the amount of Ca^{2+} accumulated. However,

addition of oxalate reduced the fluorescence intensity, presumably by decreasing intravesicular Ca^{2+} concentration, although the rate of net accumulation of Ca^{2+} actually increased. Furthermore, the fluorescence change appeared to be related to the amount of calcium bound to the membrane regardless of whether it resulted from active transport or passive binding. These findings led Vanderkooi and Martonosi (379) to suggest that the observed alteration represented the binding of accumulated calcium to the membrane and not a conformational change of a transport enzyme within the membrane.

Nakamaru and Schwartz (262) found that the addition of ATP and Ca^{2+} to sarcoplasmic reticulum caused an alteration in the absorption intensity of bromcresol purple. Thus, a rapid shift to shorter wavelength and its reversal to longer wavelength were induced by ATP alone and ATP and Ca^{2+}, respectively. They suggested that the former change was due to the formation of the Michaelis complex whereas the latter was attributable to the formation of the phosphorylated intermediate (262).

Although some of the above-mentioned changes in conformational probes may arise, to some extent, from structural changes in the ATPase, none of these studies provide solid evidence that the ATPase enzyme undergoes a specific conformational change that is directly coupled with the translocation of calcium across the membrane. However, a recent observation by Coan and Inesi (34) does suggest such an alteration.

C. Evidence for Molecular Motion: Proposal for a Rotatory Model

Recent studies by Tonomura and co-workers provide substantial evidence that the functional movement of the ATPase molecule during ATP hydrolysis can be reconciled by a simple rotatory model such as that described in section IV, *B1*.

Tonomura and Morales (371) examined how the accessibility of enzyme-bound nitroxide-maleimide spin labels to externally presented ascorbate changed with changing enzyme states of the ATPase. By varying the composition of the reaction mixture, the spin-labeled enzyme could be held in different states, such as the free enzyme (E), the enzyme-ATP complex (EATP), and the phosphorylated enzyme (EP) (see sect. VII). An ascorbate-quenching technique (178) distinguished the internal localization of the label, since the spin-label signals of the internally located label were quenched more slowly by externally applied ascorbate than were those of the externally located label. As would be predicted, solubilization of the membrane by Triton X-100 abolished the slowly quenched component. Kinetic analysis of the quenching profiles of the EPR spectrum revealed that in different enzyme states the relative amounts of the slowly and rapidly quenched fractions were different, thus allowing semiquantitative determination (percent of total label) of internal localization of labels at a given enzyme state. Tonomura and Morales (371) found that both states EP and EATP exhibited greater amounts of internal localization of labels than state E. States EP and EATP were distinguishable when the quenching

profiles of lightly and heavily labeled preparations of the membrane (0.9 and 4.0 mol/mol of ATPase, respectively) were compared. Lightly labeled preparations gave 69 and 33% of internal localization in states EP and EATP, respectively, whereas a heavily labeled preparation exhibited 57 and 75% of internal localization in states EP and EATP, respectively. The observed percentages were in good agreement with those calculated on the assumption that the appropriately distributed, spin-labeled thiol groups at the outer surface rotated internally when the enzyme formed the intermediate complexes EP and EATP.

Yamamoto and Tonomura (414) examined whether the difference in enzyme states was associated with change in the accessibility of the lysine residues of the enzyme 2,4,6-trinitrobenzenesulfonate (TBS), which by itself did not permeate the membrane (19a, 358) and did not inhibit ATPase activity. Any one of the four enzyme states was imposed on the ATPase: E, EATP, ECa, or EP. Symbols E, EATP, and EP corresponded essentially to those given by Tonomura and Morales (371) and ECa represented the enzyme-calcium complex (see sect. v and vii). The sarcoplasmic reticulum vesicles were allowed to react with TBS while the enzyme was kept in one of the above states, and the amount of trinitrophenyl (TNP)-protein complex formed was determined. Yamamoto and Tonomura (414) found that the maximal amounts of TNP incorporated into the protein varied with varying enzyme states. Thus, about 2, 3, 1, and 3 mol lysine were modified per mole of ATPase, when the enzyme was at states E, ECa, EATP, and EP, respectively. The number of the modified lysine residues, after the enzyme states were altered from one to the other, was always equal to or smaller than the sum of the number of the modified lysines at each state, indicating that some of these modifiable lysine residues were common to different enzymatic states. Yamamoto and Tonomura (414) interpreted these findings as the result of the rotatory movement of the ATPase molecule about an axis in the plane of the membrane during ATP hydrolysis.

These observations made by Tonomura and co-workers (371, 414) that the alteration in the enzymatic state resulted in change in the region of the enzyme reactive with externally presented agents lent support to the adequacy of the rotatory-carrier mechanism, which was discussed in detail in section iv, *B1*. Evidence in support of rotatory molecular motion of a protein within the membrane was obtained by Takashima et al. (351a), who investigated the frequency dependence of membrane capacitance of squid axon. The relaxation times observed, i.e., 5×10^{-5} s at rest and 2×10^{-5} s during excitation, were suggested by these investigators to be explained by the limited rotation of protein molecules within the membrane.

Although such a molecular motion of the enzyme would not be thermodynamically favored (330), the rotatory model would allow us to visualize the structural and functional transitions of the ATPase that take place continuously during the translocation of calcium. However, the model was designed to explain only a limited number of observations, so that at this time it is far from a satisfactory explanation for the dynamic properties of the ATPase associated with the translocation of calcium. It would be

interesting to perform a similar assessment when dynamic properties of the submolecular structures of the ATPase are understood more clearly (see sect. XI*B*). Such an attempt was recently made by Yamamoto and Tonomura (414a), who examined the distribution of TNP incorporated into tryptic subfragments of the ATPase protein (sect. XI*B*) fixed at various enzyme states. They found that the 20,000-dalton fragment Ib undergoes the most significant change in the number of TNP molecules incorporated with changing enzyme states, whereas the 30,000-dalton fragment Ia undergoes a less prominent change.

D. Effect of Antibodies Against ATPase on Calcium Transport

Further understanding of the mode of functional movement of the ATPase within the membrane can be gained from studies of the effects of anti-ATPase antibodies. Martonosi and Fortier (237) found that sheep or guinea pig antisera against the purified ATPase of rabbit inhibited calcium transport by rabbit sarcoplasmic reticulum, but had only a slight effect on the Ca^{2+}-dependent ATPase activity. This effect was caused by a complement-induced damage of the membrane, which led to massive leakage of Ca^{2+}. In fact, the inhibitory effect of antisera on the calcium transport was abolished when antisera were treated at 56°C for 30 min to inactivate complement. The ^{125}I-labeled purified anti-ATPase immunoglobulin G (IgG) was able to bind to the sarcoplasmic reticulum membranes and to the purified ATPase both in the presence and absence of complement. In the Ouchterlony test, in the presence of Triton X-100, anti-ATPase sera gave no precipitin lines with sarcoplasmic reticulum and the ATPase, probably due to the limited diffusion of the antigen. These findings led Martonosi and Fortier (237) to suggest that the anti-ATPase antibodies, which were able to bind to the ATPase in the absence of complement, did not inhibit calcium transport when complement was absent. However, these authors failed to present conclusive evidence that the binding of purified anti-ATPase IgG to the membrane *did not* induce inhibition of the calcium transport.

Sumida and Sasaki (343) found that a γ-globulin fraction partially purified from rabbit sera immunized against purified Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from chicken skeletal muscle significantly inhibited the active calcium transport by sarcoplasmic reticulum of chicken muscle. The γ-globulin fraction contained antibodies that combined with the purified ATPase and the membrane ATPase, as revealed by the binding of ^{131}I-labeled antibodies and by immunoprecipitation test after electrophoresis in SDS. Binding of the antibodies to the membrane did not cause passive leakage of Ca^{2+}, nor did it affect the ATPase activity, but active calcium transport was markedly inhibited by the binding of antibodies. Thus, at saturating levels of the antibodies, the rate of calcium uptake was only 30% that of the membrane treated with normal sera. The extent of inhibition of calcium transport was not affected by heating of the antibody at 56°C for 30 min. These observations led Sumida and Sasaki (343) to conclude that the binding of the anti-ATPase antibodies, but not complement, to the mem-

branes of sarcoplasmic reticulum produced a profound inhibition of the active calcium transport without altering the ATPase activity and passive permeability of the membrane to Ca^{2+}. It is not clear, however, what caused the apparent uncoupling of the transport, in which the translocation of calcium was suppressed while the ATP hydrolysis was maintained. The conclusion of these authors contrasts sharply with that of Martonosi and Fortier (237), who did not find an effect of antibodies, and the reason for this discrepancy remains to be clarified. However, the observation of Sumida and Sasaki (343) was compatible with the rotatory-carrier mechanism (see sect. IV, *B1* and XII*C*), although a molecular motion more complex than the simple rotation of the enzyme must be assumed to reconcile these findings.

Dutton et al. (59) examined effects of anti-2,4-dinitrophenyl antibodies on the calcium transport and ATP hydrolysis by sarcoplasmic reticulum. Details of these studies are described next in section XII*E*.

E. Interpretation by Mobile-Pore Model

Other attempts to interpret the mechanism of calcium transport employ a mobile-pore model, in which calcium is assumed to be translocated through a protein-lined channel that spans the membrane (see sect. IV, *B3*). The Na^+-K^+-dependent ATPase is thought to exhibit this type of mechanism, largely on the basis of suggestive data that this ATPase may form a dimer, although no direct evidence exists (see sect. XI*D*).

The most popular model of this type is that proposed to explain the coupling between proton translocation and the ATPase reaction in the mitochondrial membranes. This model assumed that an ATPase protein complex (F_1) on the outer surface of the inner mitochondrial membranes is connected to the membrane through another factor (F_0) that possesses a pore (well) that transports protons (301, 302). A variant of this model was proposed for the calcium transport of sarcoplasmic reticulum by Racker (301, 303), who assumed that calcium could be transported through a channel formed by the ATPase, which was connected to an intramembranous pore formed by proteolipid in a manner analogous to mitochondrial F_0. A similar model was proposed by Martonosi (230) and Shamoo and Goldstein (323a). It was suggested that the 20,000-dalton subfragment of the ATPase that exhibits ionophore activity for Ca^{2+} (see section XI*C*) might function as a channel or a pocket for calcium.

Dutton et al. (59) prepared sarcoplasmic reticulum vesicles in which 2,4-[^{3}H]dinitrophenylcadaverine was covalently attached to the ATPase by transglutaminase and examined the effect of anti-2,4-dinitrophenyl antibodies on the calcium transport reaction. They found that neither the ATPase nor calcium transport was affected by the binding of antibodies to the membrane and suggested that the calcium transport did not involve significant molecular motion of the ATPase. These investigators contended that such observations were consistent with the aggregate rearrangement mechanism (330), a variant form of the pore model, in which the transport

proteins are proposed to exist as aggregates of two or more similar or identical polypeptide chains (see also sect. IV, *B3*). However, it remains premature to draw this conclusion from these observations since the molecular movement coupled with calcium translocation presumably occurred even when part of the ATPase molecule was immobilized by the binding of an antibody. In view of recent findings (326, 338) that the substructures of the ATPase enzyme are separable (sect. XI*B*), it is possible that binding of antibodies to the substructure containing the phosphorylation site does not suppress the movement of the substructure containing the calcium-binding site.

Although the mobile-pore mechanism is characterized by a minimal movement of the ATPase molecule, in which the polar group of the enzyme remains outside the membrane during the cation translocation (sect. IV, *B3*), this model is not always suitable for envisioning the continuous transition of the conformational and functional properties of the enzyme. The latter are presumed to occur during the translocation of the calcium-binding site and the subsequent change in the affinity for cations (sect. VII*D*). Indeed, a much more complicated molecular motion must be assumed if the mobile-pore model is to be applied to interpret the observations made by Tonomura and Morales (371) and Yamamoto and Tonomura (414) discussed in section XII*C*.

F. Remaining Problems

We have documented the pros and cons of two different approaches to the explanation of the molecular motion of a carrier protein during cation translocation, one of the central issues in modern bioenergetics. So far, each of these models is partially satisfactory in interpreting the experimental results. We expressed our preference for the rotatory-carrier mechanism, but it is evident that both models are constructed on fragmentary information and still do not represent a complete interpretation for the molecular mechanism of energy transduction in chemiosmotic coupling. More accurate information must be accumulated before a more realistic model can be devised. Such a model has to satisfy several prerequisites. For instance, the model has to explain why the proteinous structure within the membrane, presumably representing the main body of the ATPase, is preferentially located in the cytoplasmic leaflet of the membrane (sect. II*D*). Furthermore, other features such as submolecular distribution of key functions within the enzyme (sect. XI, *B* and *C*) and the enzyme interactions with lipids have to be considered. More important, such a model should explain the reaction sequence of the elementary steps of the ATPase and calcium transport, and the mechanisms of their coupling, as discussed in detail in sections VI–X. Construction of a molecular model should in turn help provide a better understanding of the molecular mechanism of energy transduction in active calcium transport by sarcoplasmic reticulum.

XIII. SIMILARITY AMONG ADENOSINETRIPHOSPHATASES OF CATION TRANSPORT AND MUSCLE CONTRACTION

This section is designed to compare the molecular mechanisms of the Ca^{2+}-dependent ATPase, the Na^+-K^+-dependent ATPase, and myosin ATPase in order to point out similarities in the basic mechanisms of molecular operation in two major energy-transducing systems: cation transport and muscle contraction.

A. Na^+-K^+-Dependent ATPase versus Ca^{2+}-Dependent ATPase

The transport of Na^+ and K^+ across the plasma membrane, like Ca^{2+} transport into the sarcoplasmic reticulum, is coupled to the hydrolysis of ATP. In the former system, the amounts of Na^+ and K^+ transported and ATP hydrolyzed are stoichiometrically maintained, and the vectorial properties of the movement of monovalent cations are strictly kept. Skou (332) found that a Na^+-K^+-dependent ATPase is functional in the active transport of these cations. During the ATP hydrolysis catalyzed by this ATPase, the terminal phosphate of ATP is transferred to the enzyme (E) to form an acylphosphoprotein intermediate (EP) (332, 333). This phosphorylation was found to occur at the carboxyl group of aspartate in the enzyme (14). These enzymatic properties of the Na^+-K^+-dependent ATPase are clearly similar to those of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum (see sect. VI*E*). Formation of EP is stimulated by Na^+ inside the membrane; its decomposition is enhanced by K^+ outside the membrane. By a rough analogy, therefore, the roles of Ca^{2+} and Mg^{2+} in calcium transport by sarcoplasmic reticulum would be analogous to those of Na^+ and K^+ in the active transport of Na^+ and K^+, if the sidedness of the membrane in the former is inverted, although there is no direct evidence that Mg^{2+} serves as a counterion for calcium transport (sect. V, *D4*) in the same way as K^+ does for sodium transport.

The reaction mechanism of the Na^+-K^+-dependent ATPase has been extensively studied by many investigators, including Albers, Post, Tonomura, and co-workers (2, 294, 318, 320, 365, 367). Several observations indicate the existence of two types of phosphorylated intermediate. One, which is formed at high Mg^{2+} concentration, cannot react with ADP to form ATP and decomposes rapidly after the addition of K^+. The other, which is formed at lower Mg^{2+} concentration, is capable of forming ATP from ADP. Several investigators presented different views on the chemical characteristics of the two phosphoenzymes and their position in the reaction sequence of the elementary steps. Kanazawa et al. (157) and Fukushima and Tonomura (91, 92) provided evidence to support the view that these two classes of phosphorylated enzyme represented EP complexed with ADP (E_P^{ADP}) and EP with no ADP, and that the former preceded the latter. Both were placed in the following reaction sequence:

$$E + ATP \rightleftharpoons E_1ATP \rightarrow E_2ATP \rightleftharpoons E_P^{ADP} \rightleftharpoons EP + ADP \rightarrow E + P_i + ADP \quad (7)$$

According to this scheme, the first phosphorylated intermediate E_P^{ADP} was formed via two types of the enzyme-ATP complex, E_1ATP and E_2ATP. This reaction sequence was similar to that in the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum, in which the phosphorylated intermediate is formed via ${}_1E_{ATP}^{Ca_2}$ and ${}_2E_{ATP}^{Ca_2}$ (see sect. VII*A*). High concentrations of ATP stimulate ATPase activity (87, 99, 310) probably by accelerating the step $E_1ATP \rightarrow E_2ATP$ (157), an effect similar to that in the Ca^{2+}-dependent ATPase (sect. IX*A*). Further, the binding of Na^+ and ATP to the enzyme was shown by Kanazawa et al. (157) to occur in a random sequence, like that of Ca^{2+} and ATP to the Ca^{2+}-dependent ATPase (sect. VII*A*). There is evidence suggesting an essential role of phospholipids in the decomposition of EP in the Na^+-K^+-dependent ATPase (101, 353), in line with the observations in the Ca^{2+}-dependent ATPase (sect. XI*A*).

The reversal of the calcium transport was found to be coupled to the synthesis of ATP (sect. X). Essentially similar observations were made by Glynn et al. (95, 100) and Lant and Whittam (190), who found that the reversal of the cation distribution across the erythrocyte membrane by application of K^+ and Na^+ to the inside and outside, respectively, resulted in the synthesis of ATP from ADP and P_i. Employing the fragmented membranes of a Na^+-K^+ transport system in which no concentration gradient of cations was established, Post and co-workers (296, 297, 352) demonstrated that EP was formed by the reaction with P_i in the presence of a large amount of Mg^{2+}, and subsequent additions of Na^+ and ADP resulted in the net synthesis of an amount of ATP equal to that of EP (296).

The circulatory-carrier mechanism proposed by Shaw (sect. IV*A*) was accepted until recently as a popular model in explaining the coupling of the Na^+-K^+-dependent ATPase with the active transport of Na^+ and K^+. However, recent observations indicated that this model is not always adequate in explaining the mechanism of active transport of Na^+ and K^+ across the membrane. For instance, Fukushima and Tonomura (92, 367) indicated that simultaneous binding of Na^+ and K^+ to the enzyme occurred, during both the formation and the decomposition of EP. Hoffman and Tosteson (120), Garrahan and Garay (94), and Chipperfield and Whittam (32) indicated that the transport of Na^+ and K^+ was achieved only when these cations were bound to the enzyme on the interior and the exterior of the membrane, respectively. The circulatory-carrier model of Shaw might be adequate in explaining the molecular mechanism of the calcium transport by sarcoplasmic reticulum (see sect. XII*A*), but it is difficult to interpret the role of Mg^{2+} in the Ca^{2+}-dependent ATPase (sect. VII*D*) by this model.

B. Myosin ATPase versus Cation Transport ATPases

Muscle contraction is another system in which the mechanism of energy

transduction is relatively well understood. Contraction of myofibrils is achieved by the sliding of actin filaments past myosin filaments, a process that is coupled with the hydrolysis of ATP. The reaction of myosin with F-actin consists of the following three processes: *1*) binding of myosin to F-actin; *2*) movement of myosin to translocate F-actin; *3*) dissociation of F-actin from myosin. These three processes may correspond to the three elementary steps in the cation transport (see sect. IV*C*): recognition, translocation, and release of cations, respectively. In this system, decomposition of the myosin-phosphate-ADP complex, formed as an intermediate of the reaction of myosin with ATP, is accelerated by F-actin, and the resulting ATP hydrolysis is coupled to the molecular movement of the myosin cross-bridge, which contains the active site of the ATPase, to generate contraction. The accelerated ATP hydrolysis, which is also called "actomyosin type of ATPase" represents an essential enzymic process in muscle contraction.

The reaction sequence of the myosin ATPase was extensively investigated by Tonomura, Trentham, Taylor, and their co-workers (354, 362, 372, 373). Although several issues remain unanswered (354a), most investigators basically agree on the mechanism of the energy-transducing process in myosin ATPase. Different symbols are employed by different groups; however, the reaction mechanism of Tonomura and co-workers with their own symbols (145, 362, 368, 369) is represented as follows:

$$\mathrm{M + ATP} \rightleftharpoons \mathrm{M_1ATP} \rightleftharpoons \mathrm{M_2ATP} \rightleftharpoons \mathrm{M_P^{ADP}} \rightarrow {}^{o}\mathrm{M + ADP + P_i} \rightarrow \mathrm{M + ADP + P_i} \quad (8)$$

where M represents the head of myosin that is responsible for the energy transduction; M_P^{ADP} is the complex of myosin, phosphate, and ADP; and ${}^{o}M$ is the species of myosin that does not form M_P^{ADP} on addition of ATP. The existence of M_P^{ADP} was proposed by Tonomura and co-workers to explain a rapid burst of P_i when the initial phase of the reaction was stopped by addition of TCA. The rapid, initial burst of P_i was suggested to result from the rapid formation of a TCA-unstable intermediate (M_P^{ADP}) that exhibited an extremely low rate of turnover (362, 370).

Like Na^+-K^+-dependent and Ca^{2+}-dependent ATPases, whose phosphorylated intermediate is formed via two types of the enzyme-ATP complex, myosin ATPase exhibits two types of enzyme-ATP complex, M_1ATP and M_2ATP (366). The M_1ATP is in quasi-equilibrium with M + ATP, while the rate constant for the backward reaction between M_1ATP and M_2ATP is much smaller than that of the forward reaction. Thus, the key intermediates (E_P^{ADP}, EP + ADP, and M_P^{ADP}) are formed via two kinds of enzyme-ATP complex, where ATP binds loosely at first and then very tightly to the enzymes (sect. IX*A* and XIII*A*).

The reversibility of the step between M_P^{ADP} and M_2ATP was suggested earlier by Nakamura and Tonomura (265), based on the studies of the exchange reaction between P_i and ATP in the initial phase of the reaction. Direct evidence for the reversal of this process was obtained by Bagshaw and Trentham (10) and Inoue et al. (145). The latter authors utilized the

KCl-jump method since the equilibrium between M_2ATP and M_P^{ADP} is highly dependent on KCl concentration. Thus, in the presence of ATP at a concentration lower than that of myosin, a rapid decrease in the KCl concentration after the completion of the initial burst of P_i at high KCl concentration resulted in ATP synthesis. Reversibility of the ATPase reaction ($M_2ATP \rightleftharpoons M + ADP + P_i$) was also demonstrated by Mannherz et al. (219). Arata et al. (4) calculated the thermodynamic parameters ΔS^o and ΔH^o of the equilibrium between M_2ATP and M_P^{ADP} and obtained values of +16 to 19 eu/mol and +4.1–4.4 kcal/mol, respectively. These values are comparable to those of the equilibrium step between E_2ATP and E_P^{ADP} in the Na^+-K^+-dependent ATPase reaction: +15.6 eu/mol and +4.3 kcal/mol, respectively (93). Also, the formation of M_P^{ADP} from M + ATP or from M + P_i + ADP in the myosin ATPase reaction was accompanied by a great increase in ΔS^o and ΔH^o (4), as was the case in the formation of EP from E and P_i in Ca^{2+}-dependent ATPase (see sect. x*C*). These observations indicate that myosin ATPase can be reversed in a manner essentially analogous to the transport ATPases and that the thermodynamic characteristics of the formation of key intermediates among these different reactions are basically similar.

The most conspicuous difference between the myosin ATPase and the transport ATPases is the mode by which phosphate is attached to the key intermediates, M_P^{ADP}, E_P^{ADP}, and EP (366). The complex of myosin, phosphate, and ADP (M_P^{ADP}) is readily decomposed by the addition of TCA, as mentioned above, whereas termination of the transport ATPase reactions with TCA results in the formation of stable phosphorylated intermediates (E_P^{ADP} and EP) in which phosphate is covalently attached to the enzyme (see sect. VII*A* and XIII*A*). However, the striking similarity in the thermodynamic properties of the phosphate-bound intermediates of the myosin and transport ATPases suggests a resemblance in their molecular structure.

C. Similarity in Key Energy-Transducing Processes

In the active transport of Ca^{2+} by sarcoplasmic reticulum, the translocation of Ca^{2+} is coupled to the formation of the key intermediates as shown in the following equation (see also sect. VII):

$$_2E_{ATP}^{Ca^o} \rightleftharpoons E_P^{Ca^i} + ADP^o$$

This step is in rapid equilibrium and is considered to be closely associated with the molecular movement of the transport ATPase (sect. XII), and recent observations by Tonomura and Morales (371) and Yamamoto and Tonomura (414) indicate that there is a rotatory movement of this protein within the membrane (see sect. XII*C*).

Arata and Tonomura (5) suggested that the isometric tension development of glycerinated muscle fiber in myosin ATPase required the existence of a rapid equilibrium between the key intermediates:

$$M_2ATP \rightleftharpoons M_P^{ADP}$$

Thus, this step was suggested to be coupled to the change in the orientation of the head of myosin (362, 366). In particular, models proposed by A. F. Huxley and Simmons (123) and H. E. Huxley (126) to explain muscular contraction assumed rotatory and hinging motions, respectively, of the heads of myosin molecules coupled with sliding of the filaments.

Therefore, the apparent similarity in the molecular mode of coupling between the chemical reactions (a rapid equilibrium between the key intermediates) and the molecular motion of the enzyme (rotatory movement) indicates that the mechanisms of energy-transducing processes in active cation transport and muscle contraction are essentially similar. Such a working hypothesis, originally proposed by Tonomura and co-workers (366, 369), would be useful in elucidating the molecular basis of energy transduction, since the observations made in one field are expected to be applied to issues in the other, resulting in mutual progress in both fields. It is hoped that these methods of approach will enable us to obtain a better understanding of the molecular basis for energy transduction in general.

XIV. CONCLUDING REMARKS

This review outlines characteristics of the molecular mechanism by which Ca^{2+} is actively transported across the membranes of the sarcoplasmic reticulum, with emphasis on the following major topics: *1*) structural features of ATPase protein within the membrane (sect. II and XI); *2*) the reaction mechanism of Ca^{2+}-dependent ATPase (sect. VI–IX); *3*) coupling between elementary steps of ATPase and calcium transport (sect. VIII); *4*) ATP synthesis and reversal of calcium transport (sect. X); and *5*) functional movement of the ATPase molecule within the membrane (sect. IV and XII). As mentioned earlier (sect. I*B*), the membranes of sarcoplasmic reticulum provide one of the most suitable systems for examining these important characteristics in elucidating the bioenergetics of active cation transport. These studies prove that the key energy-transducing mechanism in this membrane system is associated with the Ca^{2+}-induced phosphorylation of the ATPase enzyme by ATP, which is accompanied by a profound change in the conformation of the enzyme molecule.

Based on a large body of accumulated knowledge, we present the justification for the two major models, the rotatory-carrier versus mobile-pore models, to explain at the molecular level the mechanism of active calcium transport across the membranes of sarcoplasmic reticulum (sect. XII). In addition, we point to the similarity in the elementary steps of the reactions and the thermodynamic properties of the key intermediary steps in the ATPase of this membrane to those of the Na^+-K^+-dependent ATPase and myosin ATPase (sect. XIII).

We thank the many investigators in this field who made their reprints, preprints, and unpublished observations available to us. This work was supported by research grants from the Ministry of Education, Science and Culture of Japan, the Japan Society for the Promotion of Science, Muscular Dystrophy Association, Inc., and Japan Heart Fund.

REFERENCES

1. AFFOLTER, H., M. CHIESI, R. DABROWSKA, AND E. CARAFOLI. Calcium regulation in heart cells. The interaction of mitochondrial and sarcoplasmic reticulum with troponin-bound calcium. *European J. Biochem.* 67: 389–396, 1976.
2. ALBERS, R. W. The (sodium plus potassium)-transport ATPase. In: *The Enzymes of Biological Membranes*, edited by A. Martonosi. New York: Plenum, 1976, vol. 3, p. 283–301.
3. ALLEN, G., AND N. M. GREEN. A 31-residue tryptic peptide from the active site of the [Ca^{++}]-transporting adenosine triphosphatase of rabbit sarcoplasmic reticulum. *Fed. European Biochem. Soc. Letters* 63: 188–192, 1976.
4. ARATA, T., A. INOUE, AND Y. TONOMURA. Thermodynamic and kinetic parameters of elementary steps in the reaction of H-meromyosin adenosinetriphosphatase. Remarkably large increase in the standard entropy for formation of the reactive H-meromyosin-phosphate-adenosinediphosphate complex. *J. Biochem.* 78: 277–286, 1975.
5. ARATA, T., Y. MUKOHATA, AND Y. TONOMURA. Structure and function of the two heads of the myosin molecule. VI. ATP hydrolysis, shortening, and tension development of myofibrils. *J. Biochem.* 82: 801–812, 1977.
6. ASHLEY, C. C., D. G. MOISESCU, AND R. M. ROSE. Aequorin-light and tension responses from bundles of myofibrils following a sudden change in free calcium. *J. Physiol., London* 241: 104P–106P, 1974.
7. ASHLEY, C. C., AND E. B. RIDGWAY. Simultaneous recording of membrane potential, calcium transient and tension in single muscle fibres. *Nature* 219: 1168–1169, 1968.
8. ASHLEY, C. C., AND E. B. RIDGWAY. On the relationships between membrane potential, calcium transient and tension in single barnacle muscle fibres. *J. Physiol., London* 209: 105–130, 1970.
9. AUGUSTIN, J., AND W. HASSELBACH. Studies on the fluorescence of 1-anilino-8-naphthalenesulfonate by the membranes of the sarcoplasmic reticulum. *European J. Biochem.* 35: 114–121, 1973.
10. BAGSHAW, C. R., AND D. R. TRENTHAM. The reversibility of adenosine triphosphate cleavage by myosin. *Biochem. J.* 133: 323–328, 1973.
11. BAIRD, G. D., AND S. V. PERRY. The inhibitory action of relaxing-factor preparations in the myofibrillar adenosine triphosphatase. *Biochem. J.* 77: 262–271, 1960.
12. BARLOGIE, B., W. HASSELBACH, AND M. MAKINOSE. Activation of calcium efflux by ADP and inorganic phosphate. *Fed. European Biochem. Soc. Letters* 12: 267–268, 1971.
13. BASKIN, R. J., AND D. W. DEAMER. Comparative ultrastructure and calcium transport in heart and skeletal muscle microsomes. *J. Cell Biol.* 43: 610–617, 1969.
14. BASTIDE, F., G. MEISSNER, S. FLEISCHER, AND R. L. POST. Similarity of the active site of phosphorylation of the adenosine triphosphatase for transport of sodium and potassium ions in kidney to that for transport of calcium ions in the sarcoplasmic reticulum of muscle. *J. Biol. Chem.* 248: 8385–8391, 1973.
15. BENDALL, J. R. Effect of the 'Marsh factor' on the shortening of muscle fibre models in the presence of adenosine triphosphate. *Nature* 170: 1058–1060, 1952.
16. BENDALL, J. R. Muscle-relaxing factors. *Nature* 181: 1188–1190, 1958.
17. BENNETT, H. S., AND K. R. PORTER. An electron microscope study of sectioned breast muscle of the domestic fowl. *Am. J. Anat.* 93: 61–105, 1953.
18. BERNE, R. M. Intracellular localization of skeletal-muscle relaxing factor. *Biochem. J.* 83: 364–368, 1962.
19. BOLAND, R., A. MARTONOSI, AND T. W. TILLACK. Developmental changes in the composition and function of sarcoplasmic reticulum. *J. Biol. Chem.* 249: 612–623, 1974.

19a. BONSALL, R. W., AND S. HUNT. Reactivity of the human erythrocyte membrane to sodium trinitrobenzenesulfonate. *Biochim. Biophys. Acta* 249: 281–284, 1971.

20. BOWMAN, W. C., AND M. W. NOTT. Actions of sympathomimetic amines and their antagonists on skeletal muscle. *Pharmacol. Rev.* 21: 27–72, 1969.
21. BOZLER, E. Relaxation in extracted muscle fibers. *J. Gen. Physiol.* 38: 149–159, 1954.
22. BOZLER, E. Binding of calcium and magnesium by the contractile elements. *J. Gen. Physiol.* 38: 735–742, 1954.
23. CARSTEN, M. E. The cardiac calcium pump. *Proc. Natl. Acad. Sci. US* 52: 1456–1462, 1964.
24. CARSTEN, M. E., AND W. F. H. M. MOMMAERTS. The accumulation of calcium ions by sarcotubular vesicles. *J. Gen. Physiol.* 48: 183–197, 1964.
25. CARVALHO, A. P. Binding of cations by microsomes from rabbit skeletal muscle. *J. Cellular Physiol.* 67: 73–84, 1966.
26. CARVALHO, A. P. Calcium-binding properties of sarcoplasmic reticulum as influenced by ATP, caffeine, quinine, and local anesthetics. *J. Gen. Physiol.* 52: 622–642, 1968.
27. CARVALHO, A. P., AND B. LEO. Effects of ATP on the interaction of Ca^{++}, Mg^{++}, and K^{+} with fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle. *J. Gen. Physiol.* 50: 1327–1352, 1967.
28. CARVALHO, M. G. C., D. G. DE SOUZA, AND L. DE MEIS. On a possible mechanism of energy conservation in sarcoplasmic reticulum membrane. *J. Biol. Chem.* 251: 3629–3636, 1976.
29. CHAMPEIL, P., F. BASTIDE, C. TAUPIN, AND C. M. GARY-BOBO. Spin labelled sarcoplasmic reticulum vesicles: Ca^{2+}-induced spectral changes. *Fed. European Biochem. Soc. Letters* 63: 270–272, 1976.
30. CHEVALLIER, J., AND R. A. BUTOW. Calcium binding to the sarcoplasmic reticulum of rabbit skeletal muscle. *Biochemistry* 10: 2733–2737, 1971.
31. CHIPPERFIELD, A. R., AND R. WHITTAM. Ouabain binding to the sodium pump. *Nature* 242: 62–63, 1973.
32. CHIPPERFIELD, A. R., AND R. WHITTAM. Evidence that ATP is hydrolyzed in a one-step reaction of the sodium pump. *Proc. Roy. Soc., London, Ser. B* 187: 269–280, 1974.
33. COAN, C. R., AND G. INESI. Ca^{2+} dependent effect of acetylphosphate on spin-labeled sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 71: 1283–1288, 1976.
34. COAN, C. R., AND G. INESI. Ca^{2+}-dependent effect of ATP on spin-labeled sarcoplasmic reticulum. *J. Biol. Chem.* 252: 3044–3049, 1977.
35. COFFEY, R. L., E. LANGWINSKA, M. OLIVER, AND A. MARTONOSI. The mechanism of ATP hydrolysis by sarcoplasmic reticulum. *Arch. Biochem. Biophys.* 170: 37–48, 1975.
36. COHEN, A., AND Z. SELINGER. Calcium binding

properties of sarcoplasmic reticulum membranes. *Biochim. Biophys. Acta* 183: 27–35, 1969.
37. COLE, H. A., AND S. V. PERRY. The phosphorylation of troponin I from cardiac muscle. *Biochem. J.* 149: 525–533, 1975.
38. DAVIS, D. G., AND G. INESI. Protein nuclear magnetic resonance studies of sarcoplasmic reticulum membranes. Correlation of the temperature-dependent Ca^{2+} efflux with a reversible structural transition. *Biochim. Biophys. Acta* 241: 1–8, 1971.
39. DEAMER, D. W. Isolation and characterization of a lysolecithin adenosine triphosphatase complex from lobster muscle microsomes. *J. Biol. Chem.* 248: 5477–5485, 1973.
40. DEAMER, D. W., AND R. J. BASKIN. Ultrastructure of sarcoplasmic reticulum preparations. *J. Cell Biol.* 42: 296–307, 1969.
41. DE BOLAND, A. R., R. L. JILKA, AND A. N. MARTONOSI. Passive Ca^{2+} permeability of phospholipid vesicles and sarcoplasmic reticulum membranes. *J. Biol. Chem.* 250: 7501–7510, 1975.
42. DEGANI, C., AND P. D. BOYER. A borohydride reduction method for characterization of the acyl phosphate linkage in proteins and its application to sarcoplasmic reticulum adenosine triphosphatase. *J. Biol. Chem.* 248: 8222–8226, 1973.
43. DE MEIS, L. Ca^{2+} uptake and acetyl phosphatase of skeletal muscle microsomes. Inhibition by Na^+, K^+, Li^+, and adenosine triphosphate. *J. Biol. Chem.* 244: 3733–3739, 1969.
44. DE MEIS, L. Activation of Ca^{2+} uptake by acetyl phosphate in muscle microsomes. *Biochim. Biophys. Acta* 172: 343–344, 1969.
45. DE MEIS, L. Allosteric inhibition by alkali ions of the Ca^{2+} uptake and adenosine triphosphatase activity of skeletal muscle microsomes. *J. Biol. Chem.* 246: 4764–4773, 1971.
46. DE MEIS, L. Phosphorylation of membranous protein of the sarcoplasmic reticulum. Inhibition by Na^+ and K^+. *Biochemistry* 11: 2460–2465, 1972.
47. DE MEIS, L. Regulation of steady state level of phosphoenzyme and ATP synthesis in sarcoplasmic reticulum vesicles during reversal of the Ca^{2+} pump. *J. Biol. Chem.* 251: 2055–2062, 1976.
48. DE MEIS, L., AND M. G. C. CARVALHO. Role of the Ca^{2+} concentration gradient in the adenosine 5′-triphosphate-inorganic phosphate exchange catalyzed by sarcoplasmic reticulum. *Biochemistry* 13: 5032–5038, 1974.
49. DE MEIS, L., AND M. G. C. CARVALHO. On the sidedness of membrane phosphorylation by Pi and ATP synthesis during reversal of the Ca^{2+} pump of sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 251: 1413–1417, 1976.
50. DE MEIS, L., M. G. C. CARVALHO, AND M. M. SORENSON. ATP $\rightleftharpoons$ P_i exchange catalyzed by sarcoplasmic reticulum without a Ca^{++} concentration gradient. In: *Concepts of Membranes in Regulation and Excitation*, edited by M. R. Silva and G. Suarez-Kurtz. New York: Raven, 1975, p. 7–19.
51. DE MEIS, L., AND M. C. F. DE MELLO. Substrate regulation of membrane phosphorylation and of Ca^{2+} transport in the sarcoplasmic reticulum. *J. Biol. Chem.* 248: 3691–3701, 1973.
52. DE MEIS, L., AND W. HASSELBACH. Acetyl phosphate as substrate for Ca^{2+} uptake in skeletal muscle microsomes. *J. Biol. Chem.* 246: 4759–4763, 1971.
53. DE MEIS, L., W. HASSELBACH, AND R. D. MACHADO. Characterization of calcium oxalate and calcium phosphate deposits in sarcoplasmic reticulum vesicles. *J. Cell Biol.* 62: 505–509, 1974.
54. DE MEIS, L., AND H. MASUDA. Phosphorylation of the sarcoplasmic reticulum membrane by orthophosphate through two different reactions. *Biochemistry* 13: 2057–2062, 1974.
55. DE MEIS, L., AND M. M. SORENSON. ATP $\leftrightarrow$ P_i exchange and membrane phosphorylation in sarcoplasmic reticulum vesicles: activation by silver in the absence of a Ca^{2+} concentration gradient. *Biochemistry* 14: 2739–2744, 1975.
56. DUGGAN, P. F., AND A. MARTONOSI. Sarcoplasmic reticulum. IX. The permeability of sarcoplasmic reticulum membranes. *J. Gen. Physiol.* 56: 147–167, 1970.
56a. DUPONT, Y. Kinetics and regulation of sarcoplasmic reticulum ATPase. *European J. Biochem.* 72: 185–190, 1977.
57. DUPONT, Y., S. C. HARRISON, AND W. HASSELBACH. Molecular organization in the sarcoplasmic reticulum membrane studied by X-ray diffraction. *Nature* 244: 555–558, 1973.
58. DUPONT, Y., AND W. HASSELBACH. Structural changes in sarcoplasmic reticulum membrane induced by SH reagents. *Nature New Biol.* 246: 41–44, 1973.
59. DUTTON, A., E. D. REES, AND S. J. SINGER. An experiment eliminating the rotating carrier mechanism for the active transport of Ca ion in sarcoplasmic reticulum membranes. *Proc. Natl. Acad. Sci. US* 73: 1532–1536, 1976.
60. EBASHI, S. A granule-bound relaxation factor in skeletal muscle. *Arch. Biochem. Biophys.* 76: 410–423, 1958.
61. EBASHI, S. Calcium binding and relaxation in the actomyosin. *J. Biochem.* 48: 150 151, 1960.
62. EBASHI, S. Calcium binding activity of vesicular relaxing factor. *J. Biochem.* 50: 236–244, 1961.
63. EBASHI, S. Excitation-contraction coupling. *Ann. Rev. Physiol.* 38: 293–313, 1976.
64. EBASHI, S., AND F. EBASHI. Removal of calcium and relaxation in actomyosin systems. *Nature* 194: 378–379, 1962.
65. EBASHI, S., AND M. ENDO. Ca ion and muscle contraction. *Progr. Biophys. Mol. Biol.* 18: 123–183, 1968.
66. EBASHI, S., M. ENDO, AND I. OHTSUKI. Control of muscle contraction. *Quart. Rev. Biophys.* 2: 351–384, 1969.
67. EBASHI, S., AND F. LIPMANN. Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. Cell Biol.* 14: 389–400, 1962.
68. ELETR, S., AND G. INESI. Phospholipid orientation in sarcoplasmic membranes: spin-label ESR and proton NMR studies. *Biochim. Biophys. Acta* 282: 174–179, 1972.
69. ENDO, M. Entry of a dye into the sarcotubular system of muscle. *Nature* 202: 1115–1116, 1964.
70. ENDO, M. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* 57: 71–108, 1977.
71. ENDO, M., AND J. R. BLINKS. Inconstant association of aequorin luminescence with tension during calcium release in skinned muscle fibres. *Nature New Biol.* 246: 218–221, 1973.
72. ENDO, M., M. TANAKA, AND Y. OGAWA. Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature* 228: 34–36, 1970.
73. ENGLAND, P. J. Correlation between contraction and phosphorylation of the inhibitory subunit of troponin in perfused rat heart. *Fed. European Biochem. Soc. Letters* 50: 57–60, 1975.
74. ENTMAN, M. L., E. P. BORNET, K. KANIIKE, M.

A. GOLDSTEIN, T. E. NELSON, T. W. FUTCH, AND A. SCHWARTZ. Association of glycogenolysis with cardiac sarcoplasmic reticulum. *J. Biol. Chem.* 251: 3140–3146, 1976.

75. ENTMAN, M. L., J. L. HANSEN, AND J. W. COOK, JR. Calcium metabolism in cardiac microsomes incubated with lanthanum ion. *Biochem. Biophys. Res. Commun.* 35: 258–264, 1969.
76. ENTMAN, M. L., T. R. SNOW, D. FREED, AND A. SCHWARTZ. Analysis of calcium binding and release by canine cardiac relaxing system (sarcoplasmic reticulum). The use of specific inhibitors to construct a two-component model for calcium binding and transport. *J. Biol. Chem.* 248: 7762–7772, 1973.
77. FABIATO, A., AND F. FABIATO. Relaxing and inotropic effects of cyclic AMP on skinned cardiac cells. *Nature* 253: 556–558, 1975.
78. FANBURG, B. L., AND S. MATSUSHITA. Phosphorylated intermediate of ATPase of isolated cardiac sarcoplasmic reticulum. *J. Mol. Cellular Cardiol.* 5: 111–115, 1973.
79. FIEHN, W., AND W. HASSELBACH. The effect of diethylether upon the function of the vesicles of sarcoplasmic reticulum. *European J. Biochem.* 9: 574–578, 1969.
80. FIEHN, W., AND W. HASSELBACH. The effect of phospholipase A on the calcium transport and the role of unsaturated fatty acids in ATPase activity of sarcoplasmic vesicles. *European J. Biochem.* 13: 510–518, 1970.
81. FIEHN, W., AND A. MIGALA. Calcium binding to sarcoplasmic membranes. *European J. Biochem.* 20: 245–248, 1971.
82. FOLCH, J., AND M. LEES. Proteolipides, a new type of tissue lipoproteins. Their isolation from brain. *J. Biol. Chem.* 191: 807–817, 1951.
83. FRANZINI-ARMSTRONG, C., L. LANDMESSER, AND G. PILAR. Size and shape of transverse tubule openings in frog twitch muscle fibers. *J. Cell Biol.* 64: 493 497, 1975.
84. FRATANTONI, J. C., AND A. ASKARI. Effect of monovalent cations on the adenosinetriphosphatase of skeletal muscle microsomal preparation. *Biochim. Biophys. Acta* 99: 259–269, 1965.
85. FREARSON, N. AND S. V. PERRY. Phosphorylation of the light-chain components of myosin from cardiac and red skeletal muscles. *Biochem. J.* 151: 99–107, 1975.
86. FRIEDMAN, Z., AND M. MAKINOSE. Phosphorylation of skeletal muscle microsomes by acetylphosphate. *Fed. European Biochem. Soc. Letters* 11: 69–72, 1970.
87. FROEHLICH, J. P., R. W. ALBERS, G. J. KOVAL, R. GOEBEL, AND M. BERMAN. Evidence for a new intermediate state in the mechanism of $(Na^+ + K^+)$-adenosine triphosphatase. *J. Biol. Chem.* 251: 2186–2188, 1976.
88. FROEHLICH, J. P., AND E. W. TAYLOR. Transient state kinetic studies of sarcoplasmic reticulum adenosine triphosphatase. *J. Biol. Chem.* 250: 2013–2021, 1975.
89. FROEHLICH, J. P., AND E. W. TAYLOR. Transient state kinetic effects of calcium ion on sarcoplasmic reticulum adenosine triphosphatase. *J. Biol. Chem.* 251: 2307–2315, 1976.
90. FUJINO, M., T. YAMAGUCHI, AND K. SUZAKI. 'Glycerol effect' and the mechanism linking excitation of the plasma membrane with contraction. *Nature* 192: 1159–1161, 1961.
91. FUKUSHIMA, Y., AND Y. TONOMURA. Two kinds of high energy phosphorylated intermediate, with and without bound ADP, in the reaction of N^+-K^+-dependent ATPase. *J. Biochem.* 74: 135–142, 1973.
92. FUKUSHIMA, Y., AND Y. TONOMURA. Effect of sodium and potassium ions on the elementary steps in the Na^+-K^+-dependent ATPase. *J. Biochem.* 77: 521–531, 1975.
93. FUKUSHIMA, Y., AND Y. TONOMURA. Properties of the conversion of an enzyme-ATP complex to a phosphorylated intermediate in the reaction of Na^+-K^+-dependent ATPase. *J. Biochem.* 77: 533–541, 1975.
94. GARRAHAN, P. J., AND R. P. GARAY. The distinction between sequential and simultaneous models for sodium and potassium transport. In: *Current Topics in Membrane and Transport*, edited by F. Bronner and A. Kleinzeller. New York: Academic, 1976, vol. 8, p. 29–97.
95. GARRAHAN, P. J., AND I. M. GLYNN. The incorporation of inorganic phosphate into adenosine triphosphate by reversal of the sodium pump. *J. Physiol., London* 192: 237–256, 1967.
96. GARRAHAN, P. J., A. F. REGA, AND G. L. ALONSO. The interaction of magnesium ions with the calcium pump of sarcoplasmic reticulum. *Biochim. Biophys. Acta* 448: 121–132, 1976.
97. GATTAS, C. R., AND L. DE MEIS. Ca^{2+}-dependent inhibitory effects of Na^+ and K^+ on Ca^{2+} transport in sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* 389: 506–515, 1975.
98. GLYNN, I. M. Sodium and potassium movements in human red cells. *J. Physiol., London* 134: 278–310, 1956.
99. GLYNN, I. M., AND S. J. D. KARLISH. ATP hydrolysis associated with an uncoupled sodium flux through the sodium pump: evidence for allosteric effects of intracellular ATP and extracellular sodium. *J. Physiol., London* 256: 465–496, 1976.
100. GLYNN, I. M., AND V. L. LEW. Synthesis of adenosine triphosphate at the expense of downhill cation movements in intact human red cells. *J. Physiol., London* 207: 393–402, 1970.
101. GOLDMAN, S. S., AND R. W. ALBERS. Sodium-potassium-activated adenosine triphosphatase. IX. The role of phospholipids. *J. Biol. Chem.* 248: 867–874, 1973.
102. HARDWICKE, P. M. D. Binding of antibody to the active site of the adenosine triphosphatase of sarcoplasmic reticulum. *Biochim. Biophys. Acta* 422: 357–364, 1976.
103. HARDWICKE, P. M. D. The binding of lipid to the lipid-free adenosine triphosphatase protein of sarcoplasmic reticulum. *European J. Biochem.* 62: 431–438, 1976.
104. HARDWICKE, P. M. D., AND N. M. GREEN. The effect of delipidation on the adenosine triphosphatase of sarcoplasmic reticulum. Electron microscopy and physical properties. *European J. Biochem.* 42: 183–193, 1974.
105. HARIGAYA, S., AND A. SCHWARTZ. Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. Membrane vesicles (relaxing system) and mitochondria. *Circulation Res.* 25: 781–794, 1969.
106. HASSELBACH, W. Relaxing factor and the relaxation of muscle. *Progr. Biophys. Biophys. Chem.* 14: 167–222, 1964.
107. HASSELBACH, W. The sarcoplasmic calcium pump. In: *Molecular Bioenergetics and Macromolecular Biochemistry*, edited by H. H. Weber. Berlin: Springer, 1972, p. 149–171.
108. HASSELBACH, W. Sarcoplasmic membrane ATPase. In: *The Enzymes*, edited by P. D. Boyer. New York:

Academic, 1974, vol. 10, p. 431–467.
109. HASSELBACH, W., AND L.-G. ELFVIN. Structural and chemical asymmetry of the calcium-transporting membranes of the sarcotubular system as revealed by electron microscopy. *J. Ultrastruct. Res.* 17: 598–622, 1967.
110. HASSELBACH, W., W. FIEHN, M. MAKINOSE, AND A. J. MIGALA. Calcium fluxes across isolated sarcoplasmic membranes in the presence and absence of ATP. In: *The Molecular Basis of Membrane Function*, edited by D. C. Tosteson. Englewood Cliffs, N. J.: Prentice-Hall, 1969, p. 299–316.
111. HASSELBACH, W., AND M. MAKINOSE. Die Calciumpumpe der "Erschlaffungsgrana" des Muskels und ihre Abhängigkeit von der ATP-Spaltung. *Biochem. Z.* 333: 518–528, 1961.
112. HASSELBACH, W., AND M. MAKINOSE. ATP and active transport. *Biochem. Biophys. Res. Commun.* 7: 132–136, 1962.
113. HASSELBACH, W., AND M. MAKINOSE. Über den Mechanismus des Calciumtransportes durch die Membranen des sarkoplasmatischen Reticulums. *Biochem. Z.* 339: 94–111, 1963.
114. HASSELBACH, W., AND K. SERAYDARIAN. The role of sulfhydryl groups in calcium transport through the sarcoplasmic membranes of skeletal muscle. *Biochem. Z.* 345: 159–172, 1966.
115. HASSELBACH, W., AND H. H. WEBER. Der Einfluss des MB-Factors auf die Kontraktion des Fasermodells. *Biochim. Biophys. Acta* 11: 160–161, 1953.
116. HEILBRUNN, L. V., AND F. J. WIERCINSKI. The action of various cations on muscle protoplasm. *J. Cellular Comp. Physiol.* 29: 15–32, 1947.
117. HESKETH, T. R., G. A. SMITH, M. D. HOUSLAY, K. A. MCGILL, N. J. M. BIRDSALL, J. C. METCALFE, AND G. B. WARREN. Annular lipids determine the ATPase activity of a calcium transport protein complexed with dipalmitoyllecithin. *Biochemistry* 15: 4145–4151, 1976.
118. HIDALGO, C., N. IKEMOTO, AND J. GERGELY. Role of phospholipids in the calcium-dependent ATPase of the sarcoplasmic reticulum. Enzymatic and ESR studies with phospholipid-replaced membranes. *J. Biol. Chem.* 251: 4224–4232, 1976.
119. HOFFMAN, P. G., AND D. C. TOSTESON. Active sodium and potassium transport in high potassium and low potassium sheep red cells. *J. Gen. Physiol.* 58: 438–466, 1971.
120. HOKIN, L. E. Purification and properties of the (sodium + potassium)-activated adenosine triphosphatase and reconstitution of sodium transport. *Ann. N.Y. Acad. Sci.* 242: 12–23, 1974.
121. HOLLAND, P. C., AND D. H. MACLENNAN. Assembly of sarcoplasmic reticulum. Biosynthesis of the adenosine triphosphatase in rat skeletal muscle cell culture. *J. Biol. Chem.* 251: 2030–2036, 1976.
122. HOWELL, J. N. A lesion of the transverse tubules of skeletal muscle. *J. Physiol., London* 201: 515–533, 1969.
122a. HUBBELL, W. L., AND MCCONNELL. Spin-label studies of the excitable membranes of nerve and muscle. *Proc. Natl. Acad. Sci. US* 61: 12–16, 1968.
123. HUXLEY, A. F., AND R. M. SIMMONS. Proposed mechanism of force generation in striated muscle. *Nature* 233: 533–538, 1971.
124. HUXLEY, A. F., AND R. E. TAYLOR. Local activation of striated muscle fibres. *J. Physiol., London* 144: 426–441, 1958.
125. HUXLEY, H. E. Evidence for continuity between the central elements of the triads and extracellular space in frog sartorius muscle. *Nature* 202: 1067–1071, 1964.
126. HUXLEY, H. E. The mechanism of muscular contraction. *Science* 164: 1356–1366, 1969.
127. IKEMOTO, N. The calcium binding sites involved in the regulation of the purified adenosine triphosphatase of the sarcoplasmic reticulum. *J. Biol. Chem.* 249: 649–651, 1974.
128. IKEMOTO, N. Transport and inhibitory Ca^{2+} binding sites on the ATPase enzyme isolated from the sarcoplasmic reticulum. *J. Biol. Chem.* 250: 7219–7224, 1975.
129. IKEMOTO, N. Behavior of the Ca^{2+} transport sites linked with the phosphorylation reaction of ATPase purified from the sarcoplasmic reticulum. *J. Biol. Chem.* 251: 7275–7277, 1976.
130. IKEMOTO, N., G. M. BHATNAGAR, AND J. GERGELY. Fractionation of solubilized sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 44: 1510–1517, 1971.
131. IKEMOTO, N., G. M. BHATNAGAR, B. NAGY, AND J. GERGELY. Interaction of divalent cations with the 55,000-dalton protein component of the sarcoplasmic reticulum. Studies of fluorescence and circular dichroism. *J. Biol. Chem.* 247: 7835–7837, 1972.
132. IKEMOTO, N., B. NAGY, G. M. BHATNAGAR, AND J. GERGELY. Studies on a metal-binding protein of the sarcoplasmic reticulum. *J. Biol. Chem.* 249: 2357–2365, 1974.
133. IKEMOTO, N., F. A. SRETER, AND J. GERGELY. Structural features of the surface of the vesicles of FSR – lack of functional role in Ca^{2+} uptake and ATPase activity. *Arch. Biochem. Biophys.* 147: 571–582, 1971.
134. IKEMOTO, N., F. A. SRETER, A. NAKAMURA, AND J. GERGELY. Tryptic digestion and localization of calcium uptake and ATPase activity in fragments of sarcoplasmic reticulum. *J. Ultrastruct. Res.* 23: 216–232, 1968.
135. INESI, G. p-Nitrophenyl phosphate hydrolysis and calcium ion transport in fragmented sarcoplasmic reticulum. *Science* 171: 901–903, 1971.
136. INESI, G. Active transport of calcium ion in sarcoplasmic membranes. *Ann. Rev. Biophys. Bioengineering* 1: 191–210, 1972.
137. INESI, G., AND J. ALMENDARES. Interaction of fragmented sarcoplasmic reticulum with ^{14}C-ADP, ^{14}C-ATP and ^{32}P-ATP. Effect of Ca and Mg. *Arch. Biochem. Biophys.* 126: 733–735, 1968.
138. INESI, G., AND H. ASAI. Trypsin digestion of fragmented sarcoplasmic reticulum. *Arch. Biochem. Biophys.* 126: 469–477, 1968.
139. INESI, G., J. A. COHEN, AND C. R. COAN. Two functional states of sarcoplasmic reticulum ATPase. *Biochemistry* 15: 5293–5298, 1976.
140. INESI, G., J. J. GOODMAN, AND S. WATANABE. Effect of diethyl ether on the adenosine triphosphatase activity and calcium uptake of fragmented sarcoplasmic reticulum of rabbit skeletal muscle. *J. Biol. Chem.* 242: 4637–4643, 1967.
141. INESI, G., AND W. LANDGRAF. Spin-labelling studies of fragmented sarcoplasmic reticulum. *J. Bioenergetics* 1: 355–365, 1970.
142. INESI, G., E. MARING, A. J. MURPHY, AND B. H. MCFARLAND. A study of the phosphorylated intermediate of sarcoplasmic reticulum ATPase. *Arch. Biochem. Biophys.* 138: 285–294, 1974.
143. INESI, G., M. MILLMAN, AND S. ELETR. Temperature-induced transitions of function and structure in sarcoplasmic reticulum membranes. *J. Mol. Biol.* 81: 483–504, 1973.
144. INESI, G., AND D. SCALES. Tryptic cleavage of sarco-

plasmic reticulum protein. *Biochemistry* 13: 3298–3306, 1974.

145. INOUE, A., T. ARATA, AND Y. TONOMURA. KCl jump induced formation of adenosine triphosphate from the reactive myosin-phosphate-ADP complex. *J. Biochem.* 76: 661–666, 1974.
146. ISHIKAWA, H. Formation of elaborate networks of T-system tubules in cultured skeletal muscle with special reference to the T-system formation. *J. Cell Biol.* 38: 51–66, 1968.
147. JARDETZKY, O. Simple allosteric model for membrane pumps. *Nature* 211: 969–970, 1966.
148. JILKA, R. L., A. N. MARTONOSI, AND T. W. TILLACK. Effect of the purified [Mg^{2+} + Ca^{2+}]-activated ATPase of sarcoplasmic reticulum upon the passive Ca^{2+} permeability and ultrastructure of phospholipid vesicles. *J. Biol. Chem.* 250: 7511–7524, 1975.
149. JÖBSIS, F. F., AND M. J. O'CONNER. Calcium release and reabsorption in the sartorius muscle of the toad. *Biochem. Biophys. Res. Commun.* 25: 246–252, 1966.
150. JØRGENSEN, P. L. Purification and characterization of (Na^+ + K^+)-ATPase. IV. Estimation of the purity and of the molecular weight and polypeptide content per enzyme unit in preparations from the outer medulla of rabbit kidney. *Arch. Biochem. Biophys.* 356: 53–67, 1974.

150a. JØRGENSEN, P. L. Purification and characterization of (Na^+ + K^+)-ATPase. VI. Differential tryptic modification of catalytic functions of the purified enzyme in presence of NaCl and KCl. *Biochim. Biophys. Acta* 466: 97–108, 1977.

151. JOST, P. C., O. H. GRIFFITH, R. A. CAPALDI, AND G. VANDERKOOI. Evidence for boundary lipid in membranes. *Proc. Natl. Acad. Sci. US* 70: 480–484, 1973.
152. JOST, P., O. H. GRIFFITH, R. A. CAPALDI, AND G. VANDERKOOI. Identification and extent of fluid bilayer regions in membranous cytochrome oxidase. *Biochim. Biophys. Acta* 311: 141–152, 1973.
153. KAMADA, T., AND H. KINOSHITA. Disturbances inhibited from naked surface of muscle protoplasm. *Japan. J. Zool.* 10: 469–493, 1943.
154. KANAZAWA, T. Coupling mechanism of ATP hydrolysis to active cation transport in sarcoplasmic reticulum. *Seikagaku* 44: 323–339, 1972. (In Japanese.)
155. KANAZAWA, T. Phosphorylation of solubilized sarcoplasmic reticulum by orthophosphate and its thermodynamic characteristics. The dominant role of entropy in the phosphorylation. *J. Biol. Chem.* 250: 113–119, 1975.
156. KANAZAWA, T., AND P. D. BOYER. Occurrence and characteristics of a rapid exchange of phosphate oxygens catalyzed by sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 248: 3163–3172, 1973.
157. KANAZAWA, T., M. SAITO, AND Y. TONOMURA. Formation and decomposition of a phosphorylated intermediate in the reaction of Na^+-K^+ dependent ATPase. *J. Biochem.* 67: 693–711, 1970.
158. KANAZAWA, T., S. YAMADA, AND Y. TONOMURA. ATP formation from ADP and a phosphorylated intermediate of Ca^{2+}-dependent ATPase in fragmented sarcoplasmic reticulum. *J. Biochem.* 68: 593–595, 1970.
159. KANAZAWA, T., S. YAMADA, T. YAMAMOTO, AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. V. Vectorial requirements for calcium and magnesium ions of three partial reactions of ATPase: formation and decomposition of a phosphorylated intermediate and ATP-formation from ADP and the intermediate. *J. Biochem.* 70: 95–123, 1971.
160. KATZ, A. M., J. DUNNETT, D. I. REPKE, AND W. HASSELBACH. Control of calcium permeability in the sarcoplasmic reticulum. *Fed. European Biochem. Soc. Letters* 67: 207–208, 1976.
161. KATZ, A. M., AND D. I. REPKE. Quantitative aspects of dog cardiac microsomal calcium binding and calcium uptake. *Circulation Res.* 21: 153–162, 1967.
162. KATZ, A. M., D. I. REPKE, J. E. UPSHAW, AND M. A. POLASCIK. Characterization of dog cardiac microsomes. Use of zonal centrifugation to fractionate fragmented sarcoplasmic reticulum, (Na^+ + K^+)-activated ATPase and mitochondrial fragments. *Biochim. Biophys. Acta* 205: 473–490, 1970.
163. KATZ, A. M., D. I. REPKE, J. DUNNETT, AND W. HASSELBACH. Dependence of calcium permeability of sarcoplasmic reticulum vesicles on external and internal calcium ion concentrations. *J. Biol. Chem.* 252: 1950–1956, 1977.
164. KATZ, A. M., M. TADA, AND M. A. KIRCHBERGER. Control of calcium transport in the myocardium by the cyclic AMP-protein kinase system. *Advan. Cyclic Nucleotide Res.* 5: 453–472, 1975.
165. KEPNER, G. R., AND R. I. MACEY. Red cell membrane ATPase: radiation inactivation estimates of "size." *Biochem. Biophys. Res. Commun.* 23: 202–207, 1966.
166. KEPNER, G. R., AND R. I. MACEY. Membrane enzyme systems. Molecular size determinations by radiation inactivation. *Biochim. Biophys. Acta* 163: 188–203, 1968.
167. KIELLEY, W. W., AND O. MEYERHOF. Studies on adenosinetriphosphatase of muscle. II. A new magnesium-activated adenosinetriphosphatase. *J. Biol. Chem.* 176: 591–601, 1948.
168. KIELLEY, W. W., AND O. MEYERHOF. Studies on adenosinetriphosphatase of muscle. III. The lipoprotein nature of the magnesium-activated adenosinetriphosphatase. *J. Biol. Chem.* 183: 391–401, 1950.
169. KIRCHBERGER, M. A., AND G. CHU. Correlation between protein kinase-mediated stimulation of calcium transport by cardiac sarcoplasmic reticulum and phosphorylation of a 22,000 dalton protein. *Biochim. Biophys. Acta* 419: 559–562, 1976.
170. KIRCHBERGER, M. A., AND A. RAFFO. Phosphoprotein phosphatase-catalyzed dephosphorylation of the 22,000 dalton phosphoprotein of cardiac sarcoplasmic reticulum. *J. Cyclic Nucleotide Res.* 3: 45–53, 1977.
171. KIRCHBERGER, M. A., AND M. TADA. Effects of adenosine 3′:5′-monophosphate-dependent protein kinase on sarcoplasmic reticulum isolated from cardiac and slow and fast contracting skeletal muscles. *J. Biol. Chem.* 251: 725–729, 1976.
172. KIRCHBERGER, M. A., M. TADA, AND A. M. KATZ. Adenosine 3′:5′-monophosphate-dependent protein kinase-catalyzed phosphorylation reaction and its relationship to calcium transport in cardiac sarcoplasmic reticulum. *J. Biol. Chem.* 249: 6166–6173, 1974.
173. KIRCHBERGER, M. A., M. TADA, AND A. M. KATZ. Phospholamban: a regulatory protein of the cardiac sarcoplasmic reticulum. In: *Recent Advances in Studies on Cardiac Structure and Metabolism*, edited by A. Fleckenstein and N. S. Dhalla. Baltimore: Univ. Park Press, 1975, vol. 5, p. 103–115.
174. KIRCHBERGER, M. A., M. TADA, D. I. REPKE, AND A. M. KATZ. Cyclic adenosine 3′,5′-monophosphate-dependent protein kinase stimulation of calcium uptake by canine cardiac microsomes. *J. Mol. Cellular Cardiol.* 4: 673–680, 1972.
175. KNOWLES, A. F., E. EYTAN, AND E. RACKER. Phospholipid-protein interactions in the Ca^{2+}-adenosine

triphosphatase of sarcoplasmic reticulum. *J. Biol. Chem.* 251: 5161–5165, 1976.

176. KNOWLES, A. F., AND E. RACKER. Formation of adenosine triphosphate from P_i and adenosine diphosphate by purified Ca^{++}-adenosine triphosphatase. *J. Biol. Chem.* 250: 1949–1951, 1975.
177. KNOWLES, A. F., AND E. RACKER. Properties of a reconstituted calcium pump. *J. Biol. Chem.* 250: 3538–3544, 1975.
178. KORNBERG, R. D., AND H. M. MCCONNELL. Inside-outside transitions of phospholipids in vesicle membranes. *Biochemistry* 10: 1111–1120, 1971.
179. KRASNOW, N. Effects of lanthanum and gadolinium ions on cardiac sarcoplasmic reticulum. *Biochim. Biophys. Acta* 282: 187–194, 1972.
180. KUMAGAI, H., S. EBASHI, AND F. TAKEDA. Essential relaxing factor in muscle other than myokinase and creatine phosphokinase. *Nature* 176: 166, 1955.
181. KURZMACK, M., AND G. INESI. The initial phase of Ca^{2+} uptake and ATPase activity of sarcoplasmic reticulum vesicles. *Fed. European Biochem. Soc. Letters* 74: 35–37, 1977.
182. KYTE, J. Properties of the two polypeptides of sodium- and potassium-dependent adenosine triphosphatase. *J. Biol. Chem.* 247: 7642–7649, 1972.
183. KYTE, J. The reactions of sodium and potassium ion-activated adenosine triphosphatase with specific antibodies. Implications for the mechanism of active transport. *J. Biol. Chem.* 249: 3652–3660, 1974.
184. KYTE, J. Structural studies of sodium and potassium ion-activated adenosine triphosphatase. The relationship between molecular structure and the mechanism of active transport. *J. Biol. Chem.* 250: 7443–7449, 1975.
185. LAGGNER, P. A highly α-helical structure protein in sarcoplasmic reticulum membranes. *Nature* 255: 427–428, 1975.
186. LAGGNER, P., AND M. D. BARRATT. The interaction of a proteolipid from sarcoplasmic reticulum membranes with phospholipids. A spin label study. *Arch. Biochem. Biophys.* 170: 92–101, 1975.
187. LAGGNER, P., AND D. E. GRAHAM. The effect of a proteolipid from sarcoplasmic reticulum on the physical properties of artificial phospholipid membranes. *Biochim. Biophys. Acta* 433: 311–317, 1976.
188. LANDGRAF, W. C., AND G. INESI. ATP dependent conformational change in "spin labelled" sarcoplasmic reticulum. *Arch. Biochem. Biophys.* 130: 111–118, 1969.
189. LANE, L. K., J. H. COPENHAVER, JR., G. E. LINDENMAYER, AND A. SCHWARTZ. Purification and characterisation of and [^{3}H]ouabain binding to the transport adenosine triphosphatase from outer medulla of canine kidney. *J. Biol. Chem.* 248: 7197–7200, 1973.
190. LANT, A. F., AND R. WHITTAM. The influence of ions on the labelling of adenosine triphosphate in red cell ghosts. *J. Physiol., London* 199: 457–484, 1968.
191. LARAIA, P. J., AND E. MORKIN. Adenosine 3′,5′-monophosphate-dependent membrane phosphorylation. A possible mechanism for the control of microsomal calcium transport in heart muscle. *Circulation Res.* 35: 298–306, 1974.

192a. LE MAIRE, M., K. E. JØRGENSEN, H. RØIGAARD, AND J. V. MØLLER. Properties of deoxycholate solubilized sarcoplasmic reticulum Ca^{2+}-ATPase. *Biochemistry* 15: 5805–5812, 1976.

192. LE MAIRE, M., J. V. MØLLER, AND C. TANFORD. Retension of enzyme activity by detergent-solubilized sarcoplasmic Ca^{2+}-ATPase. *Biochemistry* 15: 2336–2342, 1976.
193. LEVITSKY, D. O., M. K. ALIEV, A. V. KUZMIN, T. S. LEVCHENKO, V. N. SMIRNOV, AND E. I. CHAZOV. Isolation of calcium pump system and purification of calcium ion-dependent ATPase from heart muscle. *Biochim. Biophys. Acta* 443: 468–484, 1976.
194. LIU, S. C., AND C. R. WORTHINGTON. Electron density levels of sarcoplasmic reticulum membranes. *Arch. Biochem. Biophys.* 163: 332–342, 1974.
195. LOUIS, C. F., R. BUONAFFINA, AND B. BINKS. Effect of trypsin on the proteins of skeletal muscle sarcoplasmic reticulum. *Arch. Biochem. Biophys.* 161: 83–92, 1974.
196. LOUIS, C., AND E. M. SHOOTER. The proteins of rabbit skeletal muscle sarcoplasmic reticulum. *Arch. Biochem. Biophys.* 153: 641–655, 1972.
197. LOWE, A. G. Enzyme mechanism for the active transport of sodium and potassium ions in animal cells. *Nature* 219: 934–936, 1968.
198. MACLENNAN, D. H. Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. *J. Biol. Chem.* 245: 4508–4518, 1970.
199. MACLENNAN, D. H. Isolation of a second form of calsequestrin. *J. Biol. Chem.* 249: 980–984, 1974.
200. MACLENNAN, D. H. Resolution of the calcium transport system of sarcoplasmic reticulum. *Can. J. Biochem.* 53: 251–261, 1975.
201. MACLENNAN, D. H., AND P. C. HOLLAND. Calcium transport in sarcoplasmic reticulum. *Ann. Rev. Biophys. Bioeng.* 4: 377–404, 1975.
202. MACLENNAN, D. H., AND P. C. HOLLAND. The calcium transport ATPase of sarcoplasmic reticulum. In: *The Enzymes of Biological Membranes*, edited by A. Martonosi. New York: Plenum, 1976, vol. 3, p. 221–259.
203. MACLENNAN, D. H., V. K. KHANNA, AND P. S. STEWART. Restoration of calcium transport in the trypsin-treated (Ca^{2+} + Mg^{2+})-dependent adenosine triphosphatase of sarcoplasmic reticulum exposed to sodium dodecyl sulfate. *J. Biol. Chem.* 251: 7271–7274, 1976.
204. MACLENNAN, D. H., T. J. OSTWALD, AND P. S. STEWART. Structural components of the sarcoplasmic reticulum membrane. *Ann. N. Y. Acad. Sci.* 227: 527–536, 1974.
205. MACLENNAN, D. H., P. SEEMAN, G. H. ILES, AND C. C. YIP. Membrane formation by the adenosine triphosphatase of sarcoplasmic reticulum. *J. Biol. Chem.* 246: 2702–2710, 1971.
206. MACLENNAN, D. H., AND P. T. S. WONG. Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. US* 68: 1231–1235, 1971.
207. MACLENNAN, D. H., C. C. YIP, G. H. ILES, AND P. SEEMAN. Isolation of sarcoplasmic reticulum proteins. *Cold Spring Harbor Symp. Quant. Biol.* 37: 469–477, 1973.
208. MADEIRA, V. M. C., M. C. ANTUNES-MADEIRA, AND A. P. CARVALHO. Activation energies of the ATPase activity of sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 58: 897–904, 1974.
209. MADEIRA, V. M. C., AND M. C. ANTUNES-MADEIRA. Thermotropic transitions in sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 65: 997–1003, 1975.
210. MAKINOSE, M. Die Nucleosidtriphosphat-Nucleosiddiphosphat-Transphosphorylase-Aktivität der Vesikel des sarkoplasmatischen Reticulums. *Biochem. Z.* 345: 80–86, 1966.
211. MAKINOSE, M. The phosphorylation of the membrane protein of the sarcoplasmic vesicles during active cal-

cium transport. *European J. Biochem.* 10: 74–82, 1969.
212. MAKINOSE, M. Calcium efflux dependent formation of ATP from ADP and orthophosphate by the membranes of the sarcoplasmic vesicles. *Fed. European Biochem. Soc. Letters* 12: 269–270, 1971.
213. MAKINOSE, M. Phosphoprotein formation during osmo-chemical energy conversion in the membrane of the sarcoplasmic reticulum. *Fed. European Biochem. Soc. Letters* 25: 113–115, 1972.
214. MAKINOSE, M. Possible functional states of the enzyme of the sarcoplasmic calcium pump. *Fed. European Biochem. Soc. Letters* 37: 140–143, 1973.
215. MAKINOSE, M., AND W. HASSELBACH. Der Einfluss von Oxalat auf den Calcium-Transport isolierter Vesikel des sarkoplasmatischen Reticulum. *Biochem. Z.* 343: 360–382, 1965.
216. MAKINOSE, M., AND W. HASSELBACH. ATP synthesis by the reverse of the sarcoplasmic calcium pump. *Fed. European Biochem. Soc. Letters* 12: 271–272, 1971.
217. MAKINOSE, M., AND R. THE. Calcium-Akkumulation und Nucleosidtriphosphat-Spaltung durch die Vesikel des sarkoplasmatischen Reticulum. *Biochem. Z.* 343: 383–393, 1965.
218. MALAN, N. T., R. SABBADINI, D. SCALES, AND G. INESI. Functional and structural roles of sarcoplasmic reticulum protein components. *Fed. European Biochem Soc. Letters* 60: 122–125, 1975.
219. MANNHERZ, H. G., H. SCHENCK, AND R. S. GOODY. Synthesis of ATP from ADP and inorganic phosphate at the myosin-subfragment 1 active site. *European J. Biochem.* 48: 287–295, 1974.
220. MARSH, B. B. A factor modifying muscle fibre synaeresis. *Nature* 167: 1065–1066, 1951.
221. MARSH, B. B. The effects of adenosine triphosphate on the fibre volume of a muscle homogenate. *Biochim. Biophys. Acta* 9: 247–260, 1952.
222. MARTONOSI, A. The activating effect of phospholipids on the ATP-ase activity and Ca^{++} transport of fragmented sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 13: 273–278, 1963.
223. MARTONOSI, A. Role of phospholipids in ATPase activity and Ca transport of fragmented sarcoplasmic reticulum. *Federation Proc.* 23: 913–921, 1964.
224. MARTONOSI, A. The role of phospholipids in the ATPase activity of skeletal muscle microsomes. *Biochem. Biophys. Res. Commun.* 29: 753–757, 1967.
225. MARTONOSI, A. Sarcoplasmic reticulum. IV. Solubilization of microsomal adenosine triphosphatase. *J. Biol. Chem.* 243: 71–81, 1968.
226. MARTONOSI, A. The protein composition of sarcoplasmic reticulum membranes. *Biochem. Biophys. Res. Commun.* 36: 1039–1044, 1969.
227. MARTONOSI, A. Sarcoplasmic reticulum. VII. Properties of a phosphoprotein intermediate implicated in calcium transport. *J. Biol. Chem.* 244: 613–620, 1969.
228. MARTONOSI, A. Transport of calcium by the sarcoplasmic reticulum. In: *Metabolic Pathways*, edited by L. E. Hokin. New York: Academic, 1972, vol. 6, p. 317–349.
229. MARTONOSI, A. Biochemical and clinical aspects of sarcoplasmic reticulum function. In: *Current Topics in Membranes and Transport*, edited by F. Bronner and A. Kleinzeller. New York: Academic, 1972, vol. 3, p. 83–197.
230. MARTONOSI, A. The mechanism of calcium transport in sarcoplasmic reticulum. In: *Calcium Transport in Contraction and Secretion*, edited by E. Carafoli, F. Clementi, W. Drabikowski, and A. Margreth. Amsterdam: North-Holland, 1975, p. 313–327.
231. MARTONOSI, A. Membrane transport during development in animals. *Biochim. Biophys. Acta* 415: 311–333, 1975.
232. MARTONOSI, A., R. BOLAND, AND R. A. HALPIN. The biosynthesis of sarcoplasmic reticulum membranes and the mechanism of calcium transport. *Cold Spring Harbor Symp. Quant. Biol.* 37: 455–468, 1973.
233. MARTONOSI, A., J. DONLEY, AND R. A. HALPIN. Sarcoplasmic reticulum. III. The role of phospholipids in the adenosine triphosphatase activity and Ca^{++} transport. *J. Biol. Chem.* 243: 61–70, 1968.
234. MARTONOSI, A., J. R. DONLEY, A. G. PUCELL, AND R. A. HALPIN. Sarcoplasmic reticulum. XI. The mode of involvement of phospholipids in the hydrolysis of ATP by sarcoplasmic reticulum membranes. *Arch. Biochem. Biophys.* 144: 529–540, 1971.
235. MARTONOSI, A., AND R. FERETOS. Sarcoplasmic reticulum. I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments. *J. Biol. Chem.* 239: 648–658, 1964.
236. MARTONOSI, A., AND R. FERETOS. Sarcoplasmic reticulum. II. Correlation between adenosine triphosphatase activity and Ca^{++} uptake. *J. Biol. Chem.* 239: 659–668, 1964.
237. MARTONOSI, A., AND F. FORTIER. The effect of anti-ATPase antibodies upon the Ca^{++} transport of sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 60: 382–389, 1974.
238. MARTONOSI, A., AND R. A. HALPIN. Sarcoplasmic reticulum. X. The protein composition of sarcoplasmic reticulum membranes. *Arch. Biochem. Biophys.* 144: 66–77, 1971.
239. MARTONOSI, A., E. LAGWINSKA, AND M. OLIVER. Elementary processes in the hydrolysis of ATP by sarcoplasmic reticulum membranes. *Ann. N. Y. Acad. Sci.* 227: 549–567, 1974.
240. MARTONOSI, M. A. Thermal analysis of sarcoplasmic reticulum membranes. *Fed. European Biochem. Soc. Letters* 47: 327–329, 1974.
241. MASUDA, H., AND L. DE MEIS. Phosphorylation of the sarcoplasmic reticulum membrane by orthophosphate. Inhibition by calcium ions. *Biochemistry* 12: 4581–4585, 1973.
242. McCONNELL, H. M., K. L. WRIGHT, AND B. G. McFARLAND. The fraction of the lipid in a biological membrane that is in a fluid state: a spin label assay. *Biochem. Biophys. Res. Commun.* 47: 273–281, 1972.
243. McFARLAND, B. H., AND G. INESI. Studies of solubilized sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 41: 239–243, 1970.
244. McFARLAND, B. H., AND G. INESI. Solubilization of sarcoplasmic reticulum with Triton X-100. *Arch. Biochem. Biophys.* 145: 456–464, 1971.
245. MEISSNER, G. ATP and Ca^{2+} binding by the Ca^{2+} pump protein of sarcoplasmic reticulum. *Biochim. Biophys. Acta* 298: 906 926, 1973.
246. MEISSNER, G. Isolation and characterization of two types of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* 389: 51–68, 1975.
247. MEISSNER, G., G. E. CONNER, AND S. FLEISCHER. Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+}-pump and Ca^{2+}-binding proteins. *Biochim. Biophys. Acta* 298: 246–269, 1973.
248. MEISSNER, G., AND S. FLEISCHER. Characterization of sarcoplasmic reticulum from skeletal muscle. *Biochim. Biophys. Acta* 241: 356–378, 1971.
249. MEISSNER, G., AND S. FLEISCHER. The role of phospholipid in Ca^{2+}-stimulated ATPase activity of sarco-

plasmic reticulum. *Biochim. Biophys. Acta* 255: 19–33, 1972.

250. MEISSNER, G., AND S. FLEISCHER. Ca^{2+} uptake in reconstituted sarcoplasmic reticulum vesicles. *Biochem. Biophys. Res. Commun.* 52: 913–920, 1973.
251. MEISSNER, G., AND S. FLEISCHER. Dissociation and reconstitution of functional sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 249: 302–309, 1974.
252. MERMIER, P., AND W. HASSELBACH. Comparison between strontium and calcium uptake by the fragmented sarcoplasmic reticulum. *European J. Biochem.* 69: 79–86, 1976.
253. MIGALA, A., B. AGOSTINI, AND W. HASSELBACH. Tryptic fragmentation of the calcium transport system in the sarcoplasmic reticulum. *Z. Naturforsch.* 28: 178–182, 1973.
254. MITCHELL, P. D. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol. Rev.* 41: 445–502, 1966.
255. MIYAMOTO, H., AND M. KASAI. Asymmetric distribution of calcium binding sites of sarcoplasmic reticulum fragments. In: *Proc. Japan Biophys. Soc., 15th, Hiroshima, 1976.* p. 180.
256. MORSE, P. D. II, M. RUHLIG, W. SNIPES, AND A. D. KEITH. A spin-label study of the viscosity profile of sarcoplasmic reticular vesicles. *Arch. Biochem. Biophys.* 168: 40–56, 1975.
257. MURPHY, A. J. Cross-linking of the sarcoplasmic reticulum ATPase protein. *Biochem. Biophys. Res. Commun.* 70: 160–166, 1976.
258. MURPHY, A. J. Sulfhydryl group modification of sarcoplasmic reticulum membranes. *Biochemistry* 15: 4492–4496, 1976.
259. MUSCATELLO, U., E. ANDERSSON-CEDERGREN, AND G. F. AZZONE. The mechanism of muscle-fiber relaxation. Adenosine triphosphatase and relaxing activity of the sarcotubular system. *Biochim. Biophys. Acta* 63: 55–74, 1962.
260. MUSCATELLO, U., E. ANDERSSON-CEDERGREN, G. F. AZZONE, AND A. VON DER DECKEN. The sarcotubular system of frog skeletal muscle. A morphological and biochemical study. *J. Biophys. Biochem. Cytol. Suppl.* 10: 201–218, 1961.
261. NAGAI, T., M. MAKINOSE, AND W. HASSELBACH. Der physiologische Erschlaffungsfaktor und die Muskelgrana. *Biochim. Biophys. Acta* 43: 223–238, 1960.
262. NAKAMARU, Y., AND A. SCHWARTZ. Spectrophotometric studies on the interaction of sarcoplasmic-reticulum fragments with adenosine triphosphate and calcium. *European J. Biochem.* 34: 159–168, 1973.
263. NAKAMURA, H., H. HORI, AND T. MITSUI. Conformational change in sarcoplasmic reticulum induced by ATP in the presence of magnesium ion and calcium ion. *J. Biochem.* 72: 635–646, 1972.
264. NAKAMURA, H., R. L. JILKA, R. BOLAND, AND A. N. MARTONOSI. Mechanism of ATP hydrolysis by sarcoplasmic reticulum and the role of phospholipids. *J. Biol. Chem.* 251: 5414–5423, 1976.
265. NAKAMURA, H., AND Y. TONOMURA. The presteady state of the myosin-adenosine triphosphate system. V. Evidence for a phosphate exchange reaction between adenosine triphosphate and the "reactive myosin-phosphate complex." *J. Biochem.* 63: 279–294, 1968.
266. NAKAMURA, M., AND S. OHNISHI. Organization of lipids in sarcoplasmic reticulum membrane and Ca^{2+}-dependent ATPase activity. *J. Biochem.* 78: 1039–1045, 1975.

266a. NAKAMURA, Y., AND Y. TONOMURA. Reaction mechanism of *p*-nitrophenylphosphatase of sarcoplasmic reticulum: evidence for the two kinds of phosphorylated intermediate with and without bound *p*-nitrophenol. *J. Biochem.* In press.

267. NAKAO, M., K. NAGANO, T. NAKAO, N. MIZUNO, Y. TASHIMA, M. FUJITA, H. MAEDA, AND H. MATSUDAIRA. Molecular weight of Na,K-ATPase approximated by the radiation inactivation method. *Biochem. Biophys. Res. Commun.* 29: 588–592, 1967.
268. NAKAO, M., T. NAKAO, Y. HARA, F. NAGAI, S. YAGASAKI, M. KOI, A. NAKAGAWA, AND K. KAWAI. Purification and properties of Na,K-ATPase from pig brain. *Ann. N. Y. Acad. Sci.* 242: 24–35, 1974.
269. NATORI, R. The role of myofibrils, sarcoplasma and sarcolemma in muscle contraction. *Jikeikai Med. J.* 1: 18–28, 1954.
270. OGAWA, Y. Some properties of fragmented frog sarcoplasmic reticulum with particular reference to its response to caffeine. *J. Biochem.* 67: 667–683, 1970.
271. OHMORI, F., M. TADA, N. KINOSHITA, H. MATSUO, H. SAKAKIBARA, Y. NIMURA, AND H. ABE. Effect of protein kinase modulator on cAMP-dependent protein kinase-catalyzed phosphorylation of phospholamban and stimulation of calcium transport in cardiac sarcoplasmic reticulum. In: *Recent Advances in Studies on Cardiac Structure and Metabolism*, edited by Y. Itoh and G. Rona. Baltimore: Univ. Park Press. In press.
272. OKAMOTO, Y., AND K. YAGI. Ca^{2+}-induced conformational changes of spin-labeled g_2 chain bound to myosin and the effect of phosphorylation. *J. Biochem.* 80: 111–120, 1976.
273. OPIT, L. J., AND J. S. CHARNOCK. A molecular model for a sodium pump. *Nature* 208: 471–474, 1965.
274. OSTWALD, T. J., AND D. H. MACLENNAN. Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. *J. Biol. Chem.* 249: 974–979, 1974.
275. OSTWALD, T. J., D. H. MACLENNAN, AND K. J. DORRINGTON. Effects of cation binding on the conformation of calsequestrin and the high affinity calcium-binding protein of sarcoplasmic reticulum. *J. Biol. Chem.* 249: 5867–5871, 1974.
276. OVCHINNIKOV, Y. A. Structure and membrane activity of peptide ionophores. In: *Proc. Fed. European Biochem. Soc., 8th, Amsterdam, 1972.* Amsterdam: North-Holland, 1972, vol. 28, p. 279–306.
277. OWENS, K., R. C. RUTH, AND W. B. WEGLICKI. Lipid composition of purified fragmented sarcoplasmic reticulum of the rabbit. *Biochim. Biophys. Acta* 288: 479–481, 1972.
278. PACKER, L., C. W. MEHARD, G. MEISSNER, W. L. ZAHLER, AND S. FLEISCHER. The structural role of lipids in mitochondrial and sarcoplasmic reticulum membranes. Freeze-fracture electron microscopy studies. *Biochim. Biophys. Acta* 363: 159–181, 1974.
279. PANET, R., U. PICK, AND Z. SELINGER. The role of calcium and magnesium in the adenosine triphosphatase reaction of sarcoplasmic reticulum. *J. Biol. Chem.* 246: 7349–7356, 1971.
280. PANET, R., AND Z. SELINGER. Specific alkylation of the sarcoplasmic reticulum ATPase by *N*-ethyl-[1-^{14}C]maleimide and identification of the labeled protein in acrylamide gel-electrophoresis. *European J. Biochem.* 14: 440–444, 1970.
281. PANET, R., AND Z. SELINGER. Synthesis of ATP coupled to Ca^{2+} release from sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* 255: 34–42, 1972.
282. PANG, D. C., AND F. N. BRIGGS. Reaction mechanism of the cardiac sarcotubule calcium (II) dependent aden-

osine triphosphatase. *Biochemistry* 12: 4905–4911, 1973.
283. PANG, D. C., F. N. BRIGGS, AND R. S. ROGOWSKI. Analysis of the ATP-induced conformational changes in sarcoplasmic reticulum. *Arch. Biochem. Biophys.* 164: 332–340, 1974.
284. PARDEE, A. B. Membrane transport proteins. *Science* 162: 632–637, 1968.
285. PARDEE, A. B. Biochemical studies on active transport. *J. Gen. Physiol.* 52: 279s–288s, 1968.
286. PEACHEY, L. D. The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. Cell Biol.* 25: 209–231, 1965.
287. PEACHEY, L. D., AND R. F. SCHILD. The distribution of the T-system along the sarcomeres of frog and toad sartorius muscles. *J. Physiol., London* 194: 249–258, 1968.
288. PIRES, E., S. V. PERRY, AND M. A. W. THOMAS. Myosin light-chain kinase, a new enzyme from striated muscle. *Fed. European Biochem. Soc. Letters* 41: 292–296, 1974.
289. PORTER, K. R. The sarcoplasmic reticulum. Its recent history and present status. *J. Biophys. Biochem. Cytol. Suppl.* 10: 219–226, 1961.
290. PORTER, K. R., AND G. E. PALADE. Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle cells. *J. Biophys. Biochem. Cytol.* 3: 269–299, 1957.
291. PORTZEHL, H. Die Bindung des Erschlaffungsfaktors von Marsh an die Muskelgrana. *Biochim. Biophys. Acta* 26: 373–377, 1957.
292. POST, R. L., C. HEGYVARY, AND S. KUME. Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* 247: 6530–6540, 1972.
293. POST, R. L., AND S. KUME. Evidence for an aspartyl phosphate residue at the active site of sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* 248: 6993–7000, 1973.
294. POST, R. L., S. KUME, T. TOBIN, B. ORCUTT, AND A. K. SEN. Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase. *J. Gen. Physiol.* 54: 306s–326s, 1969.
295. POST, R. L., A. K. SEN, AND A. S. ROSENTHAL. A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes. *J. Biol. Chem.* 240: 1437–1445, 1965.
296. POST, R. L., K. TANIGUCHI, AND G. TODA. Synthesis of adenosine triphosphate by Na^+,K^+-ATPase. *Ann. N. Y. Acad. Sci.* 242: 80–91, 1974.
297. POST, R. L., G. TODA, AND F. N. ROGERS. Phosphorylation by inorganic phosphate of sodium plus potassium ion transport adenosine triphosphatase. Four reactive states. *J. Biol. Chem.* 250: 691–701, 1975.
298. PUCELL, A. G., AND A. MARTONOSI. Sarcoplasmic reticulum. XIV. Acetylphosphate and carbamylphosphate as energy sources for Ca^{++} transport. *J. Biol. Chem.* 246: 3389–3397, 1971.
299. RACKER, E. Reconstitution of a calcium pump with phospholipids and a purified Ca^{++}-adenosine triphosphatase from sarcoplasmic reticulum. *J. Biol. Chem.* 247: 8198–8200, 1972.
300. RACKER, E. A new procedure for the reconstitution of biologically active phospholipid vesicles. *Biochem. Biophys. Res. Commun.* 55: 224–230, 1973.
301. RACKER, E. The tenth Hopkins memorial lecture: Reconstitution, mechanism of action and control of ion pumps. *Biochem. Soc. Trans.* 3: 785–802, 1975.
302. RACKER, E. The coupling device. In: *A New Look at Mechanisms in Bioenergetics*. New York: Academic, 1976, p. 67–87.
303. RACKER, E. Reconstitution and mechanism of action of ion pumps. In: *A New Look at Mechanisms in Bioenergetics*. New York: Academic, 1976, p. 127–152.
304. RACKER, E., T.-F. CHIEN, AND A. KANDRACH. A cholate-dilution procedure for the reconstitution of the Ca^{++} pump, ^{32}Pi-ATP exchange, and oxidative phosphorylation. *Fed. European Biochem. Soc. Letters* 57: 14–18, 1975.
305. RACKER, E., AND E. EYTAN. Reconstitution of an efficient calcium pump without detergents. *Biochem. Biophys. Res. Commun.* 55: 174–178, 1973.
306. RACKER, E., AND E. EYTAN. A coupling factor from sarcoplasmic reticulum required for the translocation of Ca^{2+} ions in a reconstituted Ca^{2+} ATPase pump. *J. Biol. Chem.* 250: 7533–7534, 1975.
307. REPKE, D. I., J. C. SPIVAK, AND A. M. KATZ. Reconstitution of an active calcium pump in sarcoplasmic reticulum. *J. Biol. Chem.* 251: 3169–3175, 1976.
308. RIDGWAY, E. B., AND C. C. ASHLEY. Calcium transient in single muscle fibers. *Biochem. Biophys. Res. Commun.* 29: 229–234, 1967.
309. RIZZOLO, L. J., M. LE MAIRE, J. A. REYNOLDS, AND C. TANFORD. Molecular weights and hydrophobicity of the polypeptide chain of sarcoplasmic reticulum calcium (II) adenosine triphosphatase and of its primary tryptic fragments. *Biochemistry* 15: 3433–3437, 1976.
310. ROBINSON, J. D. Substrate sites of the (Na^+ + K^+)-dependent ATPase. *Biochim. Biophys. Acta* 429: 1006–1019, 1976.
311. RUBIO, R., C. BAILY, AND C. VILLAR-PALASI. Effects of cyclic AMP dependent protein kinase on cardiac actomyosin: increase in Ca^{++} sensitivity and possible phosphorylation of troponin I. *J. Cyclic Nucleotide Res.* 1: 143–150, 1975.
312. RUOHO, A., AND J. KYTE. Photoaffinity labeling of the ouabain-binding site on (Na^+ + K^+) adenosinetriphosphatase. *Proc. Natl. Acad. Sci. US* 71: 2352–2356, 1974.
313. SANSLONE, W. R., H. A. BERTLAND, B. P. YU, AND E. J. MASORO. Lipid components of sarcotubular membranes. *J. Cellular Physiol.* 79: 97–102, 1971.
314. SCALES, D. J., AND G. INESI. Localization of ATPase protein in sarcoplasmic reticulum membrane. *Arch. Biochem. Biophys.* 176: 392–394, 1976.
315. SCALES, D., AND G. INESI. Assembly of ATPase protein in sarcoplasmic reticulum membranes. *Biophys. J.* 16: 735–751, 1976.
316. SCANDELLA, C. J., P. DEVAUX, AND H. M. MCCONNELL. Rapid lateral diffusion of phospholipids in rabbit sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. US* 69: 2056–2060, 1972.
317. SCHWARTZ, A., M. L. ENTMAN, K. KANIIKE, L. K. LANE, W. B. VAN WINKLE, AND E. P. BORNET. The rate of calcium uptake into sarcoplasmic reticulum of cardiac muscle and skeletal muscle. Effects of cyclic AMP-dependent protein kinase and phosphorylase *b* kinase *Biochim Biophys Acta* 426: 57–72, 1976.
318. SCHWARTZ, A., G. E. LINDENMAYER, AND J. C. ALLEN. The Na^+, K^+-ATPase membrane transport system: importance in cellular function. In: *Current Topics in Membranes and Transport*, edited by F. Bronner and A. Kleinzeller. New York: Academic, 1972, vol. 3, p. 1–82.
319. SELINGER, Z., M. KLEIN, AND A. AMSTERDAM. Properties of particles prepared from sarcoplasmic retic-

ulum by deoxycholate. *Biochim. Biophys. Acta* 183: 19–26, 1969.

320. SEN, A. K., AND R. L. POST. Stoichiometry and localization of adenosine triphosphate-dependent sodium and potassium transport in the erythrocyte. *J. Biol. Chem.* 239: 345–352, 1964.
321. SERAYDARIAN, K., AND W. F. H. M. MOMMAERTS. Density gradient separation of sarcotubular vesicles and other particulate constituents of rabbit muscle. *J. Cell Biol.* 26: 641–656, 1965.
322. SHAMOO, A. E. Isolation of a sodium-dependent ionophore from (Na^+ + K^+)-ATPase preparations. *Ann. N. Y. Acad. Sci.* 242: 389–405, 1974.
323. SHAMOO, A. E., AND R. W. ALBERS. Na^+-selective ionophoric material derived from electric organ and kidney membranes. *Proc. Natl. Acad. Sci. US* 70: 1191–1194, 1973.

323a. SHAMOO, A. E., AND D. A. GOLDSTEIN. Isolation of ionophores from ion transport systems and their role in energy transduction. *Biochim. Biophys. Acta* 472: 13–53, 1977.

324. SHAMOO, A. E., AND D. H. MACLENNAN. A Ca^{++}-dependent and -selective ionophore as part of the Ca^{++} + Mg^{++}-dependent adenosinetriphosphatase of sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. US* 71: 3522–3526, 1974.
325. SHAMOO, A. E., AND D. H. MACLENNAN. Separate effects of mercurial compounds on the ionophoric and hydrolytic functions of the (Ca^{++} + Mg^{++})-ATPase of sarcoplasmic reticulum. *J. Membrane Biol.* 25: 65–74, 1975.
326. SHAMOO, A. E., T. E. RYAN, P. S. STEWART, AND D. H. MACLENNAN. Localization of ionophore activity in a 20,000-dalton fragment of the adenosine triphosphatase of sarcoplasmic reticulum. *J. Biol. Chem.* 251: 4147–4154, 1976.
327. SHAW, T. I. *Sodium and Potassium Movements in Red Cells* (PhD Thesis). Cambridge: Cambridge Univ., 1954.
328. SHIGEKAWA, M., J. M. FINEGAN, AND A. M. KATZ. Calcium transport ATPase of canine cardiac sarcoplasmic reticulum. A comparison with that of rabbit fast skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 251: 6894–6900, 1976.
329. SHIGEKAWA, M., AND L. J. PEARL. Activation of calcium transport in skeletal muscle sarcoplasmic reticulum by monovalent cations. *J. Biol. Chem.* 251: 6947–6952, 1976.
330. SINGER, S. J. The molecular organization of membranes. *Ann. Rev. Biochem.* 43: 805–833, 1974.
331. SINGER, S. J., AND G. L. NICOLSON. The fluid mosaic model of the structure of cell membranes. *Science* 175: 720–731, 1972.
332. SKOU, J. C. Enzymatic aspects of active linked transport of Na^+ and K^+ through the cell membrane. *Progr. Biophys. Mol. Biol.* 14: 131–166, 1964.
333. SKOU, J. C. Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. *Physiol. Rev.* 45: 596–617, 1965.
334. SKOU, J. C. Sequence of steps in the (Na + K)-activated enzyme system in relation to sodium and potassium transport. *Current Topics Bioenergetics* 4: 357–398, 1971.
335. SOLARO, R. J., A. J. G. MOIR, AND S. V. PERRY. Phosphorylation of troponin I and the inotropic effect of adrenaline in the perfused rabbit heart. *Nature* 262: 615–617, 1976.
336. STEWART, P. S., AND D. H. MACLENNAN. Surface particles of sarcoplasmic reticulum membranes. Structural features of the adenosine triphosphatase. *J. Biol. Chem.* 249: 985–993, 1974.
337. STEWART, P. S., AND D. H. MACLENNAN. Isolation and characterization of tryptic fragments of the sarcoplasmic reticulum adenosine triphosphatase. *Ann. N. Y. Acad. Sci.* 264: 326–334, 1975.
338. STEWART, P. S., D. H. MACLENNAN, AND A. E. SHAMOO. Isolation and characterization of tryptic fragments of the adenosine triphosphatase of sarcoplasmic reticulum. *J. Biol. Chem.* 251: 712–719, 1976.
339. STULL, J. T., C. O. BROSTROM, AND E. G. KREBS. Phosphorylation of the inhibitor component of troponin by phosphorylase kinase. *J. Biol. Chem.* 247: 5272–5274, 1972.
340. SUKO, J. The calcium pump of cardiac sarcoplasmic reticulum. Functional alterations at different levels of thyroid state in rabbits. *J. Physiol., London* 228: 563–582, 1973.
341. SUKO, J., AND W. HASSELBACH. Characterization of cardiac sarcoplasmic reticulum ATP-ADP phosphate exchange and phosphorylation of the calcium transport adenosine triphosphatase. *European J. Biochem.* 64: 123–130, 1976.
342. SUMIDA, M., T. KANAZAWA, AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. XI. Reevaluation of the transition of ATPase activity during the initial phase. *J. Biochem.* 79: 259–264, 1976.
343. SUMIDA, M., AND S. SASAKI. Inhibition of Ca^{2+} uptake into fragmented sarcoplasmic reticulum by antibodies against purified Ca^{2+}, Mg^{2+}-dependent ATPase. *J. Biochem.* 78: 757–762, 1975.
344. SUMIDA, M., AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. X. Direct evidence for Ca^{2+} translocation coupled with formation of a phosphorylated intermediate. *J. Biochem.* 75: 283–297, 1974.
345. TADA, M., AND M. A. KIRCHBERGER. Regulation of calcium transport by cyclic AMP. A proposed mechanism for the beta-adrenergic control of myocardial contractility. *Acta Cardiologica* 30: 231–237, 1975.
346. TADA, M., M. A. KIRCHBERGER, AND A. M. KATZ. Phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* 250: 2640–2647, 1975.
347. TADA, M., M. A. KIRCHBERGER, AND A. M. KATZ. Regulation of calcium transport in cardiac sarcoplasmic reticulum by cyclic AMP-dependent protein kinase. In: *Recent Advances in Studies on Cardiac Structure and Metabolism*, edited by P.-E. Roy and N. S. Dhalla. Baltimore: Univ. Park Press, 1976, vol. 9, p. 225–239.
348. TADA, M., M. A. KIRCHBERGER, AND H.-C. LI. Phosphoprotein phosphatase-catalyzed dephosphorylation of the 22,000-dalton phosphoprotein of cardiac sarcoplasmic reticulum. *J. Cyclic Nucleotide Res.* 1: 329–338, 1975.
349. TADA, M., M. A. KIRCHBERGER, H.-C. LI, AND A. M. KATZ. Interrelationships between calcium and cyclic AMP in the mammalian heart. In: *Calcium Transport in Contraction and Secretion*, edited by E. Carafoli, F. Clementi, W. Drabikowski, and A. Margreth. Amsterdam: North-Holland, 1975, p. 373–381.
350. TADA, M., M. A. KIRCHBERGER, D. I. REPKE, AND A. M. KATZ. Stimulation of calcium transport in cardiac sarcoplasmic reticulum by adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* 249: 6174–6180, 1974.

350a. TADA, M., F. OHMORI, N. KINOSHITA, AND H. ABE. Cyclic AMP regulation of active calcium trans-

port across membranes of sarcoplasmic reticulum. Role of the 22,000-dalton protein phospholamban. *Advan. Cyclic Nucleotide Res.* In press.

351. TADA, M., F. OHMORI, Y. NIMURA, AND H. ABE. Effect of myocardial protein kinase modulator on adenosine 3':5'-monophosphate-dependent protein kinase-induced stimulation of calcium transport by cardiac sarcoplasmic reticulum. *J. Biochem.* 82: 885–892, 1977.

351a. TAKASHIMA, S., R. YANTORNO, AND R. NOVACK. Dipole moment changes and voltage dependent capacity of squid axon. *Biochim. Biophys. Acta* 469: 74–88, 1977.

352. TANIGUCHI, K., AND R. L. POST. Synthesis of adenosine triphosphate and exchange between inorganic phosphate and adenosine triphosphate in sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* 250: 3010–3018, 1975.

353. TANIGUCHI, K., AND Y. TONOMURA. Inactivation of Na^+-K^+-dependent ATPase by phospholipase-treatment and its reactivation by phospholipids. *J. Biochem.* 69: 543–557, 1971.

354. TAYLOR, E. W. Mechanism of actomyosin ATPase and the problem of muscle contraction. *Current Topics in Bioenergetics* 5: 201–231, 1973.

354a. TAYLOR, E. W., Y. TONOMURA, AND A. INOUE. Myosin ATPase action. *Trends Biochem. Sci.* 2: N32–N35, 1977.

355. TENU, J.-P., C. GHELIS, D. SAINT LEGER, J. CARRETTE, AND J. CHEVALLIER. Mechanism of an active transport of calcium. Ethoxyformylation of sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 251: 4322–4329, 1976.

356. THE, R., AND W. HASSELBACH. Properties of the sarcoplasmic ATPase reconstituted by oleate and lysolecithin after lipid depletion. *European J. Biochem.* 28: 357–363, 1972.

357. THE, R., AND W. HASSELBACH. The modification of the reconstituted sarcoplasmic ATPase by monovalent cations. *European J. Biochem.* 30: 318–324, 1972.

358. THOMPSON, T. E., C. HUANG, AND B. J. LITMAN. Bilayers and biomembranes: compositional asymmetrics indicated by surface curvature. In: *The Cell Surface in Development*, edited by A. A. Moscona. New York: Wiley, 1974, p. 1–16.

359. THORLEY-LAWSON, D. A., AND N. M. GREEN. Studies on the location and orientation of proteins in the sarcoplasmic reticulum. *European J. Biochem.* 40: 403–413, 1973.

360. THORLEY-LAWSON, D. A., AND N. M. GREEN. Separation and characterization of tryptic fragments from the adenosine triphosphatase of sarcoplasmic reticulum. *European J. Biochem.* 59: 193–200, 1975.

361. TILLACK, T. W., R. BOLAND, AND A. MARTONOSI. The ultrastructure of developing sarcoplasmic reticulum. *J. Biol. Chem.* 249: 624–633, 1974.

362. TONOMURA, Y. Reaction mechanism of myosin-ATPase. In: *Muscle Proteins. Muscle Contraction and Cation Transport* by Y. Tonomura. Tokyo: Univ. of Tokyo Press, 1972, p. 69–113.

363. TONOMURA, Y. The regulation of muscle contraction by calcium ions and protein factors. In: *Muscle Proteins, Muscle Contraction and Cation Transport* by Y. Tonomura. Tokyo: Univ. of Tokyo Press, 1972, p. 273–304.

364. TONOMURA, Y. The Ca^{2+}-Mg^{2+}-dependent ATPase and the uptake of Ca^{2+} by the fragmented sarcoplasmic reticulum. In: *Muscle Proteins, Muscle Contraction and Cation Transport* by Y. Tonomura. Tokyo: Univ. of Tokyo Press, 1972, p. 305–356.

365. TONOMURA, Y. The Na^+-K^+-dependent ATPase of membranes. In: *Muscle Proteins, Muscle Contraction and Cation Transport* by Y. Tonomura. Tokyo: Univ. of Tokyo Press, 1972, p. 357–379.

366. TONOMURA, Y. The molecular mechanism of muscle contraction and the active transport of cations. In: *Muscle Proteins, Muscle Contraction and Cation Transport* by Y. Tonomura. Tokyo: Univ. of Tokyo Press, 1972, p. 381–407.

367. TONOMURA, Y., AND Y. FUKUSHIMA. Kinetic properties of phosphorylated intermediates in the reaction of Na^+,K^+-ATPase. *Ann. N. Y. Acad. Sci.* 242: 92–105, 1974.

368. TONOMURA, Y., AND A. INOUE. The substructure of myosin and the reaction mechanism of its adenosine triphosphatase. *Mol. Cellular Biochem.* 5: 127–143, 1974.

369. TONOMURA, Y., AND A. INOUE. Energy transducing mechanism in muscle. In: *MTP Intern. Rev. Sci., Biochemistry, Ser. One. Energy Transducing Mechanism*, edited by E. Racker. London: Butterworths, 1975, vol. 3, p. 121–161.

370. TONOMURA, Y., AND S. KITAGAWA. The initial phase of actomyosin-adenosinetriphosphatase. II. Factors influencing activity. *Biochim. Biophys. Acta* 40: 135–140, 1960.

371. TONOMURA, Y., AND M. F. MORALES. Change in state of spin labels bound to sarcoplasmic reticulum with change in enzymic state, as deduced from ascorbate-quenching studies. *Proc. Natl. Acad. Sci. US* 71: 3687–3691, 1974.

372. TONOMURA, Y., AND F. OOSAWA. Molecular mechanism of contraction. *Ann. Rev. Biophys. Bioeng.* 1: 159–190, 1972.

373. TRENTHAM, D. R., J. F. ECCLESTON, AND C. R. BAGSHAW. Kinetic analysis of ATPase mechanisms. *Quart. Rev. Biophys.* 9: 217 281, 1976.

374. UCHIDA, K., W. F. H. M. MOMMAERTS, AND D. MERETSKY. Myosin in association with preparation of sarcotubular vesicles from muscle. *Biochim. Biophys. Acta* 104: 287–289, 1965.

375. VALE, M. G. P., AND A. P. CARVALHO. Utilization of X-537A to distinguish between intravesicular and membrane-bound calcium ions in sarcoplasmic reticulum. *Biochim. Biophys. Acta* 413: 202–212, 1975.

376. VALE, M. G. P., V. R. OSÓRIO, E. CASTRO, AND A. P. CARVALHO. Synthesis of adenosine triphosphate during release of intravesicular and membrane-bound calcium ions from passively loaded sarcoplasmic reticulum. *Biochem. J.* 156: 239–244, 1976.

376a. VANDERKOOI, J. M., A. IEROKOMAS, H. NAKAMURA, AND A. MARTONOSI. Fluorescence energy transfer between Ca^{2+} transport ATPase molecules in artificial membranes. *Biochemistry* 16: 1262–1267, 1977.

377. VANDERKOOI, J., AND A. MARTONOSI. Sarcoplasmic reticulum. VIII. Use of 8-anilino-1-naphthalene sulfonate as conformational probe on biological membranes. *Arch. Biochem. Biophys.* 133: 153–163, 1969.

378. VANDERKOOI, J., AND A. MARTONOSI. Sarcoplasmic reticulum. XII. The interraction of 8-anilino-1-naphthalene sulfonate with skeletal muscle microsomes. *Arch. Biochem. Biophys.* 144: 87–98, 1971.

379. VANDERKOOI, J., AND A. MARTONOSI. Sarcoplasmic reticulum. XIII. Changes in the fluorescence of 8-anilino-1-naphthalene sulfonate during Ca^{2+} transport. *Arch. Biochem. Biophys.* 144: 99–106, 1971.

380. VEGH, K., P. SPIEGLER, C. CHAMBERLAIN, AND W. F. H. M. MOMMAERTS. The molecular size of the calcium-transport ATPase of sarcotubular vesicles estimated from radiation inactivation. *Biochim. Biophys. Acta* 163: 266–268, 1968.

381. VIANNA, A. L. Interaction of calcium and magnesium

in activating and inhibiting the nucleoside triphosphatase of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* 410: 389–406, 1975.

382. WAKU, K., Y. UDA, AND Y. NAKAZAWA. Lipid composition in rabbit sarcoplasmic reticulum and occurrence of alkyl ether phospholipids. *J. Biochem.* 69: 483–491, 1971.
383. WALTER, H., AND W. HASSELBACH. Properties of the calcium-independent ATPase of the membranes of the sarcoplasmic reticulum delipidated by the nonionic detergent Triton X-100. *European J. Biochem.* 36: 110–119, 1973.
384. WARREN, G. B., M. D. HOUSLAY, J. C. METCALFE, AND N. J. M. BIRDSALL. Cholesterol is excluded from the phospholipid annulus surrounding an active calcium transport protein. *Nature* 255: 684–687, 1975.
385. WARREN, G. B., P. A. TOON, N. J. M. BIRDSALL, A. G. LEE, AND J. C. METCALFE. Reconstitution of a calcium pump using defined membrane components. *Proc. Natl. Acad. Sci. US* 71: 622–626, 1974.
386. WARREN, G. B., P. A. TOON, N. J. M. BIRDSALL, A. G. LEE, AND J. C. METCALFE. Reversible lipid titrations of the activity of pure adenosine triphosphatase-lipid complexes. *Biochemistry* 13: 5501–5507, 1974.
387. WARREN, G. B., P. A. TOON, N. J. M. BIRDSALL, A. G. LEE, AND J. C. METCALFE. Complete control of the lipid environment of membrane-bound proteins: application to a calcium transport system. *Fed. European Biochem. Soc. Letters* 41: 122–124, 1974.
388. WATANABE, S. Relaxing effects of EDTA on glycerol-treated muscle fibers. *Arch. Biochem. Biophys.* 54: 559–562, 1955.
389. WATANABE, S., AND W. SLEATOR, JR. EDTA relaxation of glycerol-treated muscle fibers, and the effect of magnesium, calcium and manganese ions. *Arch. Biochem. Biophys.* 68: 81–101, 1957.
390. WATANABE, S., Y. TONOMURA, AND H. SHIOKAWA. Mechanism of muscular contraction. II. Kinetic studies on muscle ATPase. *J. Biochem.* 40: 387–402, 1953.
391. WEBER, A. On the role of calcium in the activity of adenosine 5′-triphosphate hydrolysis by actomyosin. *J. Biol. Chem.* 234: 2764–2769, 1959.
392. WEBER, A. Energized calcium transport and relaxing factors. *Current Topics Bioenergetics* 1: 203–254, 1966.
393. WEBER, A. Regulatory mechanisms of the calcium transport system of fragmented rabbit sarcoplasmic reticulum. I. The effect of accumulated calcium on transport and adenosine triphosphate hydrolysis. *J. Gen. Physiol.* 57: 50–63, 1971.
394. WEBER, A. Regulatory mechanisms of the calcium transport system of fragmented sarcoplasmic reticulum. II. Inhibition of outflux in calcium-free media. *J. Gen. Physiol.* 57: 64–70, 1971.
395. WEBER, A., AND R. HERZ. Requirement for calcium in the synaeresis of myofibrils. *Biochem. Biophys. Res. Commun.* 6: 364–368, 1961.
396. WEBER, A., AND R. HERZ. The binding of calcium to actomyosin systems in relation to their biological activity. *J. Biol. Chem.* 238: 599–605, 1963.
397. WEBER, A., R. HERZ, AND I. REISS. On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum. *J. Gen. Physiol.* 46: 679–702, 1963.
398. WEBER, A., R. HERZ, AND I. REISS. Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. *Biochem. Z.* 345: 329–369, 1966.
399. WEBER, A., R. HERZ, AND I. REISS. The nature of the cardiac relaxing factor. *Biochim. Biophys. Acta* 131: 188–194, 1967.
400. WEBER, A., AND J. M. MURRAY. Molecular control mechanisms in muscle contraction. *Physiol. Rev.* 53: 612–673, 1973.
401. WEBER, A., AND S. WINICUR. The role of calcium in the superprecipitation of actomyosin. *J. Biol. Chem.* 236: 3198–3202, 1961.
402. WHITTAM, R. The assymetrical stimulation of a membrane adenosine triphosphatase in relation to active cation transport. *Biochem. J.* 84: 110–118, 1962.
403. WILL, H., J. BLANCK, G. SMETTAN, AND A. WOLLENBERGER. A quench-flow kinetic investigation of calcium ion accumulation by isolated cardiac sarcoplasmic reticulum. Dependence of initial velocity on free calcium ion concentration and influence of preincubation with a protein kinase, MgATP, and cyclic AMP. *Biochim. Biophys. Acta* 449: 295–303, 1976.

403a. WINEGRAD, S. The intracellular site of calcium activation of contraction in frog skeletal muscle. *J. Gen. Physiol.* 50: 77–88, 1970.

404. WORTHINGTON, C. R., AND S. C. LIU. Structure of sarcoplasmic reticulum membranes at low resolution (17 Å). *Arch. Biochem. Biophys.* 157: 573–579, 1973.
405. WRAY, H. L., R. R. GRAY, AND R. A. OLSSON. Cyclic adenosine 3′, 5′-monophosphate-stimulated protein kinase and a substrate associated with cardiac sarcoplasmic reticulum. *J. Biol. Chem.* 248: 1496–1498, 1973.
406. YAMADA, S., M. SUMIDA, AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. VIII. Molecular mechanism of the conversion of osmotic energy to chemical energy in the sarcoplasmic reticulum. *J. Biochem.* 72: 1537–1548, 1972.
407. YAMADA, S., AND Y. TONOMURA. Phosphorylation of the Ca^{2+}-Mg^{2+}-dependent ATPase of the sarcoplasmic reticulum coupled with cation translocation. *J. Biochem.* 71: 1101–1104, 1972.
408. YAMADA, S., AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. VII. Recognition and release of Ca^{2+} ions. *J. Biochem.* 72: 417–425, 1972.
409. YAMADA, S., AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. IX. Kinetic studies on the conversion of osmotic energy to chemical energy in the sarcoplasmic reticulum. *J. Biochem.* 74: 1091–1096, 1973.
410. YAMADA, S., T. YAMAMOTO, T. KANAZAWA, AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. VI. Co-operative transition of ATPase activity during the initial phase. *J. Biochem.* 70: 279–291, 1971.
411. YAMADA, S., T. YAMAMOTO, AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. III. Ca^{2+}-uptake and ATP-splitting. *J. Biochem.* 67: 789–794, 1970.
412. YAMAMOTO, T., AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. I. Kinetic studies. *J. Biochem.* 62: 558–575, 1967.
413. YAMAMOTO, T., AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. II. Intermediate formation of phosphoryl protein. *J. Biochem.* 64: 137–145, 1968.
414. YAMAMOTO, T., AND Y. TONOMURA. Chemical modification of the Ca^{2+}-dependent ATPase of sarco-

plasmic reticulum from skeletal muscle. II. Use of 2,4,6-trinitrobenzenesulfonate to show functional movements of the ATPase molecule. *J. Biochem.* 79: 693–707, 1976.

414a. YAMAMOTO, T., AND Y. TONOMURA. Chemical modification of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. III. Changes in distribution of exposed lysine residues among subfragments with change in enzymatic state. *J. Biochem.* 82: 653–660, 1977.

415. YAMAMOTO, T., A. YODA, AND Y. TONOMURA. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. IV. Hydroxamate formation from a phosphorylated intermediate and 2-hydroxy-5-nitrobenzyl hydroxylamine. *J. Biochem.* 69: 807–809, 1971.

415a. YARIV, J., I. Z. STEINBERG, A. J. KALB, R. GOLDMAN, AND E. KATCHALSKI. Permease as a rotatory carrier. *J. Theoret. Biol.* 35: 459–465, 1972.

416. YU, B. P., AND E. J. MASORO. Functioning of histidine residues in sarcotubular membrane (Ca^{++} + Mg^{++})-ATPase and Ca^{++} transport activities. *Federation Proc.* 30: Abstr. 1170, 1971.

417. YU, B. P., E. J. MASORO, AND H. A. BERTRAND. The functioning of histidine residues of sarcoplasmic reticulum in Ca^{2+} transport and related activities. *Biochemistry* 13: 5083–5087, 1974.

418. YU, B. P., E. J., MASORO, AND F. D. DEMARTINIS. Imidazole and sequestration of calcium ions by sarcoplasmic reticulum. *Nature* 216: 822–824, 1967.

419. YU, B. P., E. J. MASORO, AND T. F. MORLEY. Analysis of the arrangement of protein components in the sarcoplasmic reticulum of rat skeletal muscle. *J. Biol. Chem.* 251: 2037–2043, 1976.

420. ZUBRZYCKA, E., AND D. H. MACLENNAN. Assembly of the sarcoplasmic reticulum. Biosynthesis of calsequestrin in rat skeletal muscle cell cultures. *J. Biol. Chem.* 251: 7733–7738, 1976.