EFFECT OF pH AND TEMPERATURE UPON CALCIUM ACCUMULATION AND RELEASE BY BEEF AND RABBIT SARCOPLASMIC RETICULUM AND MITOCHONDRIA

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ABSTRACT

EFFECT OF pH AND TEMPERATURE UPON CALCIUM ACCUMULATION AND RELEASE BY BEEF AND RABBIT SARCOPLASMIC RETICULUM AND MITOCHONDRIA

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The objectives of this study were to determine the relative abilities of red and white muscle sarcoplasmic reticulum (SR) and mitochondria to accumulate and release Ca^{++} under conditions known to exist in cold shortened muscle. The SR and mitochondria were isolated immediately after slaughter from beef Sternomandibularis (red) and rabbit Longissimus dorsi (white) muscle. Isolation was accomplished by homogenization of the muscle followed by differential centrifugation. Further purification of the SR was achieved by sucrose density gradient centrifugation.

The yield of SR was 62 ± 8 µg/gram of beef muscle as compared to 180 ± 40 µg/gram for rabbit muscle. The yield of mitochondria from the two muscles was similar. However, histochemical staining for NADH-tetrazolium reductase activity showed that the beef muscle contained a much higher concentration of mitochondria. Transmission electron microscopy and SDS gel electrophoresis showed that the SR preparations were essentially free from contamination. Electron microscopy similarly showed that mitochondrial preparations

consisted primarily of mitochondria, and manometric measurement of oxygen consumption demonstrated that they contained actively respiring mitochondria.

Ca^{++} accumulation was determined by using radioactive calcium ($^{45}Ca^{++}$). After Millipore filtration, the quantity of Ca^{++} accumulated by the SR or mitochondria was determined by measuring the radioactivity remaining in the filtrate. Ca^{++} release from preloaded SR or mitochondria due to changes in pH, temperature or oxygen content of the incubation medium was determined by comparison of the Ca^{++} content of the membranes before and after imposing conditions causing Ca^{++} release.

The SR from both beef and rabbit muscle accumulated in excess of 500 nmol Ca^{++}/mg protein at pH 7.3 and 37°C. Mitochondria from both muscles accumulated more than 400 nmol of Ca^{++}/mg protein under similar conditions. Ca^{++} accumulation by both SR and mitochondrial suspensions was markedly temperature dependent. At 0°C, preparations from red or white muscle accumulated less than 80 nmol Ca^{++}/mg protein, regardless of the pH. However, chilled SR and mitochondria retained the ability to accumulate significant quantities of Ca^{++}, if the medium was warmed to 37°C.

Ca^{++} accumulation by SR and mitochondria from both red and white muscle was also observed to be sensitive to pH. Low pH values, in the range 5.0 to 5.5 reduced the Ca^{++} accumulating capacity of both SR and mitochondria to 0 to 40 nmol Ca^{++}/mg protein. Mitochondria accumulated significant

quantities of Ca^{++} at pH 6.2 and 37°C, but Ca^{++} accumumulation was maximal for both SR and mitochondria in the pH range 6.8 to 7.3

Rabbit mitochondria accumulated somewhat more Ca^{++} under anaerobic conditions than beef mitochondria, but both preparations accumulated significant amounts of Ca^{++} under anaerobic conditions or in the presence of the uncoupling agent, 2,4-dinitrophenol. This suggested that some mitochondrial Ca^{++} accumulation was supported by ATP hydrolysis. Preloaded mitochondria from both beef and rabbit muscle released only small amounts of Ca^{++} when nitrogen was bubbled through the medium. Nevertheless, the quantities released were sufficient to initiate shortening in intact muscle.

Chilling of SR and mitochondria from both beef and rabbit muscle also caused the release of small but physiologically significant quantities of Ca^{++}. On lowering the pH to 5.0, virtually all of the initial Ca^{++} load was released.

Since SR and mitochondria from red and white muscles did not differ in their response to conditions promoting cold shortening, it was concluded that cold shortening is related to the quantities of SR and mitochondria present in the muscle. The relationship of the SR and mitochondria to cold shortening was discussed, and a mechanism of cold shortening was proposed describing how postmortem muscle pH, temperature and anaerobic conditions influence the phenomenon.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

INTRODUCTION

Cold shortening routinely occurs when beef or lamb carcasses are chilled after slaughter, resulting in a significant increase in muscle toughness (Marsh et al., 1974). Meat from animals containing a high proportion of white muscles (rabbits and pigs) do not cold shorten appreciably (Locker and Hagyard, 1963; Marsh et al., 1972). Cold shortening may be avoided by delaying chilling until after the muscles have gone into rigor, usually about 16 to 24 hours postmortem.

Locker and Daines (1976) showed that the toughness associated with cold shortened meat may be avoided by rewarming the carcass to 37^{o}C during the last stages of rigor, but this procedure has not been widely accepted by the meat industry. Fat cattle are generally thought to produce high quality, tender meat. It has been suggested that this may be due to inhibition of cold shortening as a result of the excessive fat covering insulating the carcass, thus delaying chilling until the muscles have passed into rigor. Better methods for avoiding cold shortening could lead to the production of high quality tender meat from thinner cattle, which can be produced more cheaply.

Pearson et al. (1973) demonstrated that microinjections of Ca^{++} resulted in massive shortening, which was accompanied by toughening. They postulated that both cold shortening and thaw rigor are due to the ineffectiveness of the sarcoplasmic reticulum in retaining Ca^{++} ions at cold temperatures. Davey and Gilbert (1974) also theorized that the sarcoplasmic reticulum membrane releases Ca^{++} when chilled to $0^{o}C$, and is responsible for cold shortening. Buege and Marsh (1975) found that oxygen inhibited cold shortening in chilled beef strips. They postulated that anaerobic conditions cause the release of Ca^{++} from mitochondria, and at low temperatures the sarcoplasmic reticulum is unable to accumulate Ca^{++}, thus causing shortening. Buege and Marsh (1975) pointed out that red muscles contain more mitochondria than white muscles, accounting for the observation that red muscles cold shorten more extensively than white (Locker and Hagyard, 1963).

Many changes occur in cold shortened muscle, including decreases in pH, temperature, ATP and oxygen levels (Kasten-Schmidt, 1970). The ability of muscle mitochondria to accumulate Ca^{++} under these conditions has never been fully documented. Consequently, the present study was undertaken to determine the relative abilities of mitochondria and sarcoplasmic reticulum from red and white muscle to accumulate or release Ca^{++} under the influence of pH and temperature conditions known to exist in cold shortened muscle.

LITERATURE REVIEW

I. COLD SHORTENING

Cold Shortening and Rigor

Locker and Hagyard (1963) were the first to report the cold shortening phenomenon. They observed that beef *Sternomandibularis* muscle strips shortened up to 47% when chilled to $0^{o}C$ immediately postmortem. They also found that beef *Longissimus dorsi* and *Psoas major* muscle strips shortened 43% and 39%, respectively, whereas the same muscle strips from rabbit shortened only 9% and 7%. Shortening was completed by 15 to 24 hours. Minimum shortening (10% or less) was observed in the temperature range of 14^{o} to $19^{o}C$. Locker and Hagyard (1963) further observed that high temperature shortening occurred in beef *Sternomandibularis* muscle strips, which shortened by 26% at $37^{o}C$. At higher temperatures, shortening was observed to coincide with the onset of rigor mortis (rigor shortening), but cold shortening began almost immediately upon chilling (Locker and Hagyard, 1963).

Locker and Hagyard (1963) also found that upon rewarming, the cold shortening effect was reversible. Recooling caused the muscle to contract again, but both effects were diminished as postmortem time increased. The ability of the muscle to cold shorten persisted up to the onset of rigor,

although the speed and degree of shortening diminished with time (Locker and Hagyard, 1963). As pointed out by Davey and Gilbert (1975), muscle is living tissue until it enters rigor mortis. As such, it can be stimulated to contract by cold.

Busch et al. (1967) compared cold shortening with rigor shortening in beef muscle. They found that cold shortening begins much sooner than rigor shortening; that more tension develops during cold shortening; that cold shortening occurs in the presence of 5-6 mM ATP, while rigor shortening occurs only after the ATP level drops to 1 mM or less; and that cold shortening begins while the muscle pH is above 6.0, whereas rigor shortening begins at muscle pH values below 6.0.

Cold shortened muscle is significantly tougher than non-cold shortened muscle (Marsh et al., 1974; Locker and Daines, 1976), apparently due to the sarcomere shortening (Marsh and Carse, 1974; Marsh et al., 1974). It is well established that meat tenderness is influenced by the degree of sarcomere shortening (Marsh and Leet, 1966; Marsh and Carse, 1974). After the muscle enters rigor, the sarcomere length remains unchanged (Busch et al., 1972). However, Locker and Daines (1976) observed that raising the temperature to 37^{o}C in the final stages of rigor completely nullifies the toughness seen in cold shortened meat, without affecting shortening. Dayton et al. (1976) suggested that the tenderness associated with aged meat is due to endogenous

proteolytic myofibrillar degradation at the level of the Z-line. However, Locker and Daines (1976) observed that the Z-lines were intact in cold shortened samples held at 37^{o} for 7 hours. These same investigators suggested that the 37^{o}C temperature treatment may modify the actin-myosin bonds of the muscle, resulting in the improved tenderness effect. Extensive cold shortening has also been found to have a tenderizing effect on muscle (Marsh et al., 1974), apparently due to the presence of supercontracted areas alternating with extensively stretched and torn sarcomeres (Marsh et al., 1974; Weidemann et al., 1967; Hsieh et al., 1978).

Possible Mechanisms of Cold Shortening

Ca^{++} has been shown to activate muscle contraction in living muscle (Podolsky and Constantin, 1964; Podolsky, 1975). Davey and Gilbert (1974) demonstrated that chilling prerigor muscle from 15 to 0^{o}C increased the concentration of Ca^{++} by thirty to forty fold in the myofibrillar region. They concluded that chilling caused the release of Ca^{++} from the sarcoplasmic reticulum, which is the membraneous system responsible for Ca^{++} accumulation and storage in living muscle (Peachey, 1970). Martonosi and Feretos (1964) previously had shown that lower temperatures substantially reduce the activity of the Ca^{++} pump of the sarcoplasmic reticulum. Davey and Gilbert (1974) cited evidence (Gurd, 1963; Caputo, 1968) that cell membrane phospholipids undergo distinct temperature dependent phase transitions, producing membranes of changed pore size and electrical properties.

With increased porosity, the Ca^{++} pump would be less able to stem the flow of Ca^{++} from the leaky reticulum. Thus, Davey and Gilbert (1974) ascribed the variable response of different muscles to chilling as being due to the extent of development of the sarcoplasmic reticulum. Cold shortening is less likely to occur in fast acting, white muscles that are rich in sarcoplasmic reticulum (Revel, 1964) as compared to the slow red muscles which contain less sarcoplasmic reticulum (Hasselbach, 1964).

Buege and Marsh (1975) have proposed that the muscle mitochondria are involved in cold shortening. They pointed out that those muscles that are obviously red in color cold shorten extensively. Chilling of the pale muscles of the rat and rabbit provokes little or no shortening (Locker and Hagyard, 1963; Hill, 1972). Red muscles have a much higher mitochondrial content than white muscles (Gauthier, 1970). Buege and Marsh (1975) found that thin strips of beef Sternomandibularis muscle chilled to $2^{o}C$ in a nitrogen atmosphere shortened an average of 22%, but strips held in an oxygen atmosphere shortened much less. They found that the oxygen supression of cold shortening could be overcome by mitochondrial uncoupling or respiration inhibiting agents, including dinitrophenol, ruthenium red, dicumarol, and carbonyl cyanide m-chlorophenylhydrazone. These reagents had no significant effect on the length of muscle strips held at $20^{o}C$, but at $2^{o}C$ they caused up to 40% shortening. Buege and Marsh (1975) further observed that strips of rabbit Psoas

muscle (a white muscle), shortened only 3% when chilled in a nitrogen atmosphere or in the presence of ruthenium red or dinitrophenol.

Mitochondria are a significant reservoir of intracellular Ca^{++} (Popescu et al., 1976), and isolated liver mitochondria have a large capacity for respiration linked Ca^{++} accumulation (Carafoli, 1975). Isolated liver mitochondria release calcium slowly when incubated anaerobically, and more rapidly when treated with uncoupling agents (Lehninger et al., 1967). Drahota et al. (1965) have shown that liver mitochondria release Ca^{++} in the presence of respiratory inhibitors, or in the absence of a respiratory substrate. On this basis, Buege and Marsh (1975) concluded that cold shortening is a consequence of anoxia-induced Ca^{++} release from muscle mitochondria at a temperature low enough to prevent compensating Ca^{++} uptake by the temperature sensitive sarcoplasmic reticulum.

II. SARCOPLASMIC RETICULUM

Model for the Organization of Intact Sarcoplasmic Reticulum

The sarcoplasmic reticulum (SR) is an extensive intracellular membrane system of muscle cells that functions as the storage site for Ca^{++} in the resting muscle fiber (Peachey, 1970). Peachey (1970) further stated that the sarcoplasmic reticulum is the organelle responsible for the release of Ca^{++} during activation of muscle contraction, and is responsible for Ca^{++} accumulation during muscle

relaxation. Peachey (1970) proposed a model (Figure 1) for the organization of the sarcoplasmic reticulum system. According to this model, the sarcoplasmic reticulum consists of the terminal cisternae, the longitudinal tubules and the fenestrated collar. Peachey (1970) pointed out that the T-tubule system, which is a branching membrane system continuous with the cell membrane, is not a part of the SR membrane system. Peachey (1970) showed that in frog muscle, the T (transverse) tubules form regular parallel arrays that cross the myofibrils at the level of the Z band. According to the model proposed by Peachey (1970), the terminal cisternae of the SR are located on both sides of the T tubule, forming the "triad". The longitudinal tubules extend from the terminal cisternae along the myofibril and connect with adjacent longitudinal tubules in the region called the "fenestrated collar", which encircles each sarcomere of the myofibril (Peachey, 1970). Peachey (1970) also pointed out that larger or more quickly contracting muscle cells contained more extensively developed SR and T tubule membrane systems.

The Contraction-Relaxation Cycle

Huxley (1964) first observed that frog muscles soaked in ferritin prior to electron microscopic observation contained ferritin within the T tubules. Since ferritin cannot penetrate intact membranes, Huxley (1964) concluded that the T tubules are continuous with the surface membrane, and are thus responsible for carrying nerve impulses to the interior

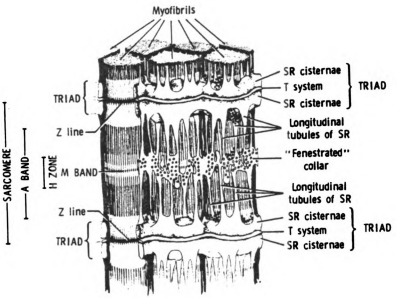

Fig. 1. Three dimensional reconstruction of the sarcoplasmic reticulum (SR) of frog sartorius muscle.

(Peachey, 1970)

of the muscle cell.

Although the SR and the T tubules are closely coupled in the region of the triad, the membranes are not continuous, and a signal must be transmitted between them at specific junctional sites (Franzini-Armstrong, 1975). Depolarization of the T tubules does cause release of Ca^{++} from the SR system, by an unknown mechanism (Podolsky, 1975). Small quantities of added Ca^{++} also cause a release of Ca^{++} from the SR, and it has been postulated that the release of small quantities of Ca^{++} from the T tubules during depolarization result in a more massive release of Ca^{++} from the SR (Podolsky, 1975; Endo, 1977).

After Ca^{++} is released into the myofibrillar region, Ca^{++} is bound to the protein troponin in the myofibrillar structure (Ebashi et al., 1969), and releases constraints on the interaction between actin and myosin, permitting muscle contraction (MacLennan, 1975). After nervous stimulation ceases, the sarcoplasmic reticulum accumulates Ca^{++} again through the action of its' Ca^{++} transport ATPase enzyme (MacLennan, 1975). MacLennan (1975) pointed out that the Ca^{++}-troponin complex has a dissociation constant of 3 μM, but the ATPase of SR has a K_m for Ca^{++} of 0.3 μM (Hasselbach, 1964). Therefore, Ca^{++} is drawn into the SR, and the muscle relaxes (MacLennan, 1975).

Characterization of Sarcoplasmic Reticulum Vesicles

Marsh (1951; 1952) was the first to recognize that a soluble fraction of a muscle homogenate could cause an

increase in the volume of myofibril preparations, which was in effect a relaxation process. This fraction was termed the "relaxation factor" and was later shown to contain sarcoplasmic reticulum vesicles (Muscatello et al., 1961; Ebashi and Lipmann, 1962). The vesicles could accumulate Ca^{++} in the presence of ATP (Hasselbach and Makinose, 1961; Ebashi and Lipmann, 1962). Weber et al. (1963) found that lowering the Ca^{++} concentration of the sarcoplasm to levels below 10^{-7} M caused muscle relaxation. These observations made it clear that the vesicles were derived from the muscle membrane responsible for in vivo muscle relaxation.

The vesicles can be isolated by differential and sucrose gradient centrifugation. Meissner (1975) isolated the vesicles on a 25-45% (w/w) linear sucrose gradient. Starting with 2000 g of rabbit muscle, he obtained about 125 mg of light SR vesicles in the 28-32% sucrose fraction, 750 mg of intermediate density vesicles in 32-39% sucrose, and 150 mg of heavy vesicles in the 39-43% sucrose fraction. The differences in density of the vesicles were due to differing phospholipid to protein ratios, the light and heavy vesicles containing 45 and 30% phospholipid, respectively. Some 90% of the protein of the light vesicles was due to the SR-ATPase protein, as determined by SDS gel electrophoresis. The heavy vesicles contained a Ca^{++} binding protein (MW 65,000) and a M_{55} protein (MW 55,000) which accounted for 25 and 5% of the protein of the vesicles, respectively. Electron micrographs showed dense material in the heavy

vesicles, but not in the light vesicles. Similar micrographs of intact SR showed dense material in the terminal cisternae, but not in the longitudinal tubules. Meissner (1975) concluded that light and heavy vesicles are derived from the longitudinal tubules and the terminal cisternae of the sarcoplasmic reticulum, respectively.

Protein Composition of the Sarcoplasmic Reticulum Vesicles

Using sodium dodecyl sulfate (SDS) gel electrophoresis, MacLennan (1975) showed that ATPase (102,000 daltons) was the predominant protein in rabbit SR vesicles. Also present were protein bands of 55,000 daltons (the high affinity Ca^{++}-binding protein), 44,000 daltons (calsequestrin), small bands with molecular weights of 30,000 and 20,000 and a proteolipid with a mobility equivalent to a molecular weight of 6,000.

Characterization of the SR-ATPase molecule

Incubation of the vesicles with trypsin cleaved the ATPase peptide to yield fragments of 55,000 and 45,000 daltons (MacLennan, 1975). The ATPase active site was identified by phosphorylation with radioactive (gamma-^{32}P) ATP; only the 55,000 dalton fragment was labelled (MacLennan, 1975). If tryptic digestion of the vesicles was continued for 30 minutes, fragments of 30,000 and 20,000 daltons were produced. The phosphorus label could be detected only in the 30,000 dalton fragment, indicating that this fragment contained the ATP hydrolytic site (MacLennan, 1975). From

amino acid analysis of the tryptic fragments and electron microscopic studies of the vesicles, MacLennan (1975) concluded that the 55,000 dalton fragment had a polar amino acid composition, contained the site for ATP hydrolysis, and was located on the exterior surface of the vesicles. The 45,000 dalton fragment was more hydrophobic, and was apparently buried in the membrane.

Shamoo et al. (1976) obtained similar fragments upon tryptic digestion of SR vesicles. Only the 20,000 dalton fragment had Ca^{++} selective ionophore activity in artificial lipid bilayers, measured as conductance changes across the lipid membrane with Ca^{++} carrying the current. The 20,000 dalton fragment was further degraded with CNBr, and fragments of less than 2,000 daltons had ionophore activity (Shamoo et al., 1976). They concluded that the ATPase and Ca^{++} ionophore sites were located in different parts of the 102,000 dalton peptide.

Characterization of Calsequestrin and High Affinity Ca^{++}-Binding Protein

Calsequestrin and the high affinity Ca^{++}-binding protein can be isolated from SR vesicles using dilute deoxycholate and salt, and can be fractionated on DEAE-cellulose, using a salt gradient between 0.0-0.7 M (MacLennan, 1975). Calsequestrin is a very acidic protein, with 146 of its' 392 amino acid residues being either glutamic or aspartic acid (MacLennan, 1975). Calsequestrin binds up to 970 nmol Ca^{++}/mg protein, with a dissociation constant of 50 μM (MacLennan,

1975). Both proteins are located on the inside of the SR vesicle membrane, since treatment of intact vesicles with proteases does not affect them (MacLennan, 1975).

Lipid Composition of the Sarcoplasmic Reticulum Vesicles

LeMaire et al. (1976) reported that the lipid fraction of the SR vesicles was composed largely of phospholipid, of which 66% was lecithin. Small quantities of cholesterol and triglyceride were also present. Several investigators (LeMaire et al., 1976; Scales and Inesi, 1976; MacLennan, 1975) have reported that the SR-ATPase polypeptide is tightly complexed with 20-30 phospholipid molecules, which are necessary for ATPase activity. LeMaire et al. (1976) found that deoxycholate removed the phospholipid from the ATPase polypeptide, but inactivated the ATPase. They further reported that the Ca^{++}-ATPase is an oligomer in the native membrane, with a molecular weight of 400,000.

Model of the Organization of the SR Vesicles

MacLennan (1972) described a model for the organization of the proteins in the SR membrane, which is shown in Figure 2. According to this model, the proteolipid and the ATPase are tightly bound proteins associated with the membrane phospholipid bilayer. The ATPase appears to have an amphipathic character. A portion, perhaps half of the molecule, is relatively nonpolar and is buried in the bilayer region of the membrane and may contain the ionophoric site. The other portion of the molecule is more polar and is located

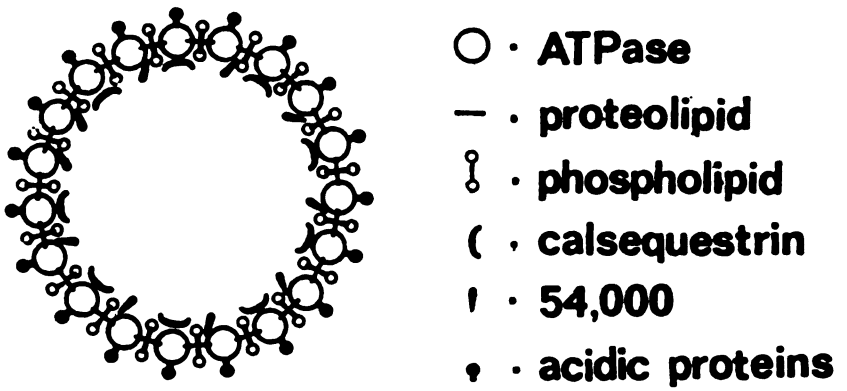

Fig. 2. A model for the arrangement of the protein and lipid components in sarcoplasmic reticulum vesicles.

(MacLennan et al., 1972)

on the exterior of the membrane, and is the site of ATP hydrolysis. The interaction of the site for ATP hydrolysis and the ionophoric site controls the transport of Ca^{++} across the membrane. The acidic proteins, calsequestrin and the high affinity Ca^{++} binding protein are only loosely bound to the membrane, perhaps through divalent salt bridges to the membrane phospholipids, and are located on the inner surface of the vesicles. They apparently function by binding the Ca^{++} that is transported inside the vesicles by the ATPase protein.

Role of the Phosphoprotein Intermediate in Ca^{++} Transport

Hasselbach and Makinose (1962) concluded that SR vesicles catalyze a transphosphorylation reaction between nucleoside triphosphates and nucleoside diphosphates. The rate of ATP-ADP exchange is dependent on the free Ca^{++} concentration, as is SR ATPase activity and calcium transport. They suggested that the ATP-ADP exchange is a partial reaction of ATP hydrolysis. Yamamoto and Tonomura (1967) discovered that there is a phosphoprotein intermediate in the reaction, whereas, Martonosi (1972) observed that this may be the connecting link among the previous observations on ATP-ADP exchange, ATPase activity, and Ca^{++} transport.

The phosphoprotein intermediate was demonstrated after incubation of vesicles with ATP-^{32}P or acetylphosphate-^{32}P, yielding protein bound ^{32}P radioactivity that was retained even after washing the vesicles repeatedly with trichloroacetic acid solution (Pucell and Martonosi, 1971). In a

review, Martonosi (1972) pointed out that the rate of phosphoprotein formation is markedly dependent on the Ca^{++} concentration of the medium in the range of 10^{-7} to 10^{-5} M Ca^{++}. This is also the range where the hydrolysis of ATP and ATP-ADP exchange are markedly activated (Yamamoto and Tonomura, 1967), if 5 mM $MgCl_2$ is also present. In the absence of Mg^{++}, about 100 times greater Ca^{++} concentrations (1-5 mM) are required to produce a similar increase in the steady state concentration of phosphoprotein (Martonosi, 1967). MacLennan (1975) also observed that the SR ATPase activity has a very precise requirement for both Mg^{++} and Ca^{++}. In the presence of an optimum concentration of Mg^{++} (5 mM), he observed that there was no ATPase activity unless about 0.3 μM Ca^{++} was also added. In the presence of 0.3 μM free Ca^{++}, there was no activity until $MgCl_2$ was added.

Mechanism of Ca^{++} Transport in Sarcoplasmic Reticulum Vesicles

Yates and Duance (1976) used the flow dialysis method to measure the kinetics of substrate binding to the SR ATPase enzyme. They concluded that the binding of MgATP and Ca^{++} may occur in a random manner, with neither substrate influencing the affinity of the enzyme for the other. The independence of Ca^{++} binding and phosphoprotein formation suggested that phosphorylation of the ATPase initiates a conformational change that leads to translocation of the Ca^{++} previously bound to specific and chemically distinct sites (Martonosi, 1972).

The ATPase activity of SR vesicles can be divided into two steps, phosphorylation and dephosphorylation of the ATPase protein (MacLennan, 1975). The substrate for phosphorylation is MgATP. The phosphorylation reaction has an absolute requirement for 0.3 μM free Ca^{++} (MacLennan, 1975). After phosphorylation, the affinity of the enzyme for Ca^{++} decreases (Berman et al., 1977) but the ATPase reaction goes to completion only after Mg^{++}-dependent dephosphorylation (Yamamoto, 1972; Garrahan et al, 1976). The SR ATPase protein of rabbit muscle contains one specific ATP site and two specific Ca^{++} sites per phosphorylation, resulting in the transport of two moles of Ca^{++} for each mole of ATP used during phosphorylation (Meissner, 1973). MacLennan (1975) postulated that Mg^{++} is the counterion for Ca^{++} transport, resulting in the release of Mg^{++}, ADP and inorganic phosphate on the membrane exterior, while Ca^{++} is released on the membrane interior. The SR membrane is freely permeable to Mg^{++}, so the Mg^{++} content of the vesicles will not limit Ca^{++} uptake (Vale, 1975).

Increasing the concentration of ADP above 1 mM progressively inhibited ATPase activity and Ca^{++} transport by isolated SR vesicles (Makinose, 1969), but the phosphoprotein concentration remained high (1.5 moles/10^6 g protein). Makinose (1969) concluded that ADP formed an unreactive complex with the ATPase phosphoprotein.

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Evidence for Two Functional States of the ATPase Protein

In the absence of Ca^{++}, rabbit SR vesicles have a low ATPase activity (basic activity), which greatly increases on addition of Ca^{++} to give the total activity (Hasselbach, 1964). The difference between the total and basic activities is known as the extra or Ca^{++}-dependent ATPase, which is coupled to Ca^{++} transport (Hasselbach, 1964). Inesi et al. (1976) found that basic ATPase activity was predominant in the low density fraction of SR vesicles obtained by density gradient centrifugation. In all other fractions, the Ca^{++}-dependent ATPase activity was predominant. In these fractions, the ratio of Ca^{++}-dependent to basic ATPase activity was temperature dependent, being about 9.0 at $40^{o}C$ and 0.5 at $4^{o}C$ (Inesi et al., 1976). Conversion of ATPase from one state to another was postulated to involve changes in the conformation of the protein and/or its' membrane environment (Inesi et al., 1976). In both states, a phosphorylated intermediate of the ATPase was formed in the presence of Ca^{++}. Hydrolysis of the phosphorylated intermediate occurred in state E_2, which is coupled to Ca^{++} transport, whereas, in state E_1 the ATPase catalyzed ATP hydrolysis (basic activity) in the absence of Ca^{++} and independently of enzyme phosphorylation (Inesi et al., 1976).

Ca^{++} Accumulation by Sarcoplasmic Reticulum Vesicles

SR vesicles may accumulate Ca^{++} under both active and passive conditions. In the absence of ATP (passive

conditions), Ca^{++} is bound on the exterior of the membrane, but in the presence of ATP, Ca^{++} is transported to the interior of the vesicle (Vale et al., 1976). Verjovski et al. (1977) concluded that SR vesicles contain low and high affinity sites for Ca^{++}, and that both increase their affinity for Ca^{++} some 3-4 fold as the pH increases from 6.1 to 8.5.

In the absence of membrane permeable Ca^{++} precipitating agents, such as oxalate or inorganic phosphate (P_i), the rate of Ca^{++} uptake by SR vesicles declines rapidly as the Ca^{++} concentration inside the vesicles increases, even if the levels of ATP and Ca^{++} in the medium are constant (Martonosi, 1972). After accumulation of 100-200 nmol Ca^{++}/mg protein, a steady state between Ca^{++} influx and Ca^{++} efflux is established. The steady state Ca^{++} flux is 50-100 times slower than the maximum initial rate of Ca^{++} uptake (Martonosi, 1972). The activity of the Ca^{++} pump is inhibited if the intravesicular free Ca^{++} concentration exceeds 2×10^{-5} M (Makinose and Hasselbach, 1965). Oxalate or phosphate, by decreasing the intravesicular free Ca^{++} concentration, permits Ca^{++} accumulation to proceed until 8,000-10,000 nmol Ca^{++}/mg protein are accumulated in the form of calcium oxalate or phosphate (Martonosi, 1972). However, the rate of Ca^{++} efflux from SR vesicles is greatly slowed in the presence of these agents (Sorenson and De Meis, 1977).

Several studies have reported that monovalent cations inhibit Ca^{++} accumulation by SR vesicles (De Meis and Hasselbach, 1971; De Meis, 1971; De Meis and De Mello, 1973). However, Shigekawa and Pearl (1976) found that K^+, Na^+, Rb^+, NH_4^+, Cs^+, Li^+, choline$^+$, and Tris$^+$ (in order of decreasing effectiveness) stimulated Ca^{++} uptake and Ca^{++}-dependent ATPase activity of SR vesicles, possibly due to an increased rate of decomposition of the phosphorylated ATPase intermediate in the presence of these ions.

The most widely used technique for determining Ca^{++} accumulation is by using $^{45}Ca^{++}$, and separating the vesicles from the media by the Millipore filtration technique (Meissner and Fleisher, 1971; Kanda, 1975). With this method, Ca^{++} accumulation is measured over a period of 10-20 minutes. As pointed out by Endo (1977), the Ca^{++} accumulating capacity of the SR measured by this method is sufficient to explain the relaxed state of muscle at steady state. However, the rate of uptake is too slow to explain the rate of relaxation in living muscle, possibly due to the nonphysiological state of the SR preparation (Endo, 1977).

Much faster rates of Ca^{++} uptake have been observed using spectrophotometric methods for monitoring Ca^{++} movement, using Ca^{++} sensitive dyes such as murexide (Reed and ByGrave, 1975), or a flow dialysis method (Mermier and Hasselbach, 1976). Mermier and Hasselbach (1976) observed that the active transport of Ca^{++} by rabbit SR vesicles was biphasic. The fast uptake phase had a $T_{1/2}$ of 2 seconds, a

maximum uptake of 80nmol Ca^{++}/mg protein, and involved active transport but no active binding of Ca^{++}. The slow uptake of Ca^{++} was due to the P_i liberated by the SR ATPase, which then aided in Ca^{++} retention, increasing the capacity of the vesicles for Ca^{++}. Sorenson and De Meis (1977) found that Ca^{++} accumulation by SR vesicles from cardiac and white skeletal muscle reached a maximum of 200-250nmol Ca^{++}/mg protein in less than a minute, followed by the release of part of the Ca^{++} over the next several minutes. Will et al. (1976) observed that preincubation of cardiac SR vesicles with a soluble cardiac cyclic AMP-dependent protein kinase, MgATP and cyclic AMP led to a significant increase in the initial rate of Ca^{++} uptake, which was measured by a quench flow spectrophotometric method. Will et al. (1976) concluded that the amount of Ca^{++} accumulated in the first 200 milliseconds was sufficient to induce relaxation in heart muscle.

Ca^{++} Release by Sarcoplasmic Reticulum Vesicles

Endo (1977) has proposed that depolarization may cause Ca^{++} release from the T-tubules which then stimulates further release of Ca^{++} from the SR. However, a variety of other conditions will lead to Ca^{++} release from isolated SR vesicles. Ca^{++} efflux from SR vesicles may occur by diffusion or by a carrier mediated mechanism (Martonosi, 1972). Release of Ca^{++} by diffusion was calculated to be 100-1,000 fold faster than that of the carrier mediated mechanism

(Martonosi, 1972).

Carrier mediated Ca^{++} efflux from SR vesicles was found to be a reversal of the ATPase mediated Ca^{++} uptake (Carvalho et al., 1976), if the medium was Ca^{++} free, or if EGTA was present (Blondin and Green, 1975). One mole of ATP is synthesized per two moles of Ca^{++} released (Makinose and Hasselbach, 1971; Carvalho et al., 1976), and the rate of Ca^{++} release is greatly increased by addition of 0.05 M ADP and 3 mM P_i (Barlogie et al., 1971).

Martonosi and Feretos (1964) observed enhanced rates of Ca^{++} efflux at alkaline pH, elevated temperature, or in the presence of sulfhydryl reagents. Kanda et al. (1977a) observed that SR vesicles released Ca^{++} when the pH was lowered to 6.2 or less, or the temperature lowered to $0^{o}C$. Addition of caffeine or changes in the electrolyte composition of the medium were found to increase SR membrane permeability, thereby increasing Ca^{++} release (Inesi and Malan, 1976). However, direct electrical stimulation of SR vesicles did not promote Ca^{++} release (Van der Kloot, 1966). Kasai and Miyamoto (1976) found that anion exchange in the medium, from methanesulfonate to chloride, caused Ca^{++} release, possibly due to SR membrane depolarization. Similar release of Ca^{++} was observed on lowering the osmotic pressure, apparently due to bursting of the SR membrane. Cation exchange had no effect on Ca^{++} efflux, but Ca^{++} accumulated in the presence of oxalate or sucrose was not released or else was released more slowly (Kasai and

Miyamoto, 1976). Meissner and McKinley (1976) concluded from similar experiments that the ion-induced changes in SR membrane permeability are due to osmotic effects, not membrane depolarization.

Effects of pH and Temperature on Ca^{++} Transport by Sarcoplasmic Reticulum

Berman et al. (1977) found that brief exposure of rabbit SR vesicles to pH values from 5.5 to 6.0 rapidly and irreversibly inactivated Ca^{++} accumulation at 25°C, but the Ca^{++} dependent ATPase activity was enhanced by 75%. At pH 5.0 to 5.5, the Ca^{++}-dependent ATPase activity was abolished, but the Ca^{++} independent-Mg^{++}-dependent ATPase activity was more resistant to the acid conditions. Berman et al. (1977) also observed that 5 mM $CaCl_2$ preserved 86% of activity of the SR for Ca^{++} uptake after incubation for 5 minutes at pH 5.9. Berman et al. (1977) observed that aged SR suspensions were more susceptible to acid inactivation, whereas, Van der Kloot (1969) found that dithiothreitol prevented any loss of activity during aging. Kanda et al. (1977a) also observed that aging reduced the ability of SR vesicles to accumulate Ca^{++}. Kanda et al. (1977a) further observed that at pH 6.2 or less, SR vesicles had reduced ability to accumulate Ca^{++}, and vesicles preloaded with Ca^{++} released part of it at pH 6.2 or below. Ca^{++} was also released when the temperature was reduced to 0°C (Kanda et al., 1977a).

La Court (1971) observed that Ca^{++} uptake by beef SR vesicles decreased as the pH dropped from 6.7 to 5.6, and was completely inhibited at pH values below 5.8 or above 7.7, or at temperatures below $5^{o}C$, regardless of the pH. However, the SR ATPase activity remained constant in the pH range of 5.6 to 6.7. La Court (1971) demonstrated that muscle lysosomal proteases are active at pH 5.5 and below. He concluded that proteolysis, together with the drop in pH and temperature, results in the loss of the Ca^{++} accumulating ability by SR of postmortem muscle.

Ca^{++} Accumulation by Sarcoplasmic Reticulum Vesicles of Postmortem Muscle

Greaser et al (1967) showed that the Ca^{++}-binding ability of porcine SR vesicles drops markedly with increasing time postmortem, and by 24 hours the isolated SR vesicles retained only 10% of their original Ca^{++} accumulating ability. Greaser et al. (1969) also observed that vesicles isolated from pale, soft and exudative porcine muscle, which undergoes a rapid postmortem pH drop, had lost their Ca^{++} accumulating ability by one hour postmortem.

Goll et al. (1971) demonstrated that rabbit SR lost its ability to accumulate Ca^{++} just prior to rigor onset, but the ATPase activity of the vesicles remained constant until the development of maximum tension. Nauss and Davies (1966) found that postmortem tension development in frog Sartorius muscle was accompanied by an increased rate of

Ca^{++} efflux, even in the presence of ATP. Goll et al. (1971) suggested three possible causes for the loss of Ca^{++} accumulating ability by postmortem SR vesicles, which included proteolysis, postmortem pH decline, and postmortem loss of ATP. They finally concluded that proteolysis was the principle factor responsible for loss of the Ca^{++} accumulating ability.

Comparison of Sarcoplasmic Reticulum from Red and White Muscles

Red muscles yield 0.7-1.4 mg SR protein/g tissue (Patriarca and Carafoli, 1969; Harigaya et al., 1968), which is less than the yield of 2.5-4.0 mg SR protein/g tissue for white muscles (Schiaffino et al., 1970). Several investigators have reported that the rate and extent of Ca^{++} uptake by SR vesicles of red muscles is less than that for white muscles (Table 1) (Sreter and Gergely, 1964; Harigaya and Schwartz, 1969; Sreter, 1969; Samaha and Gergely, 1965a; Samaha and Gergely, 1965b; Katz and Repke, 1967). Sreter (1969) reported that SR vesicles from red rabbit muscle accumulated a maximum of 40 nmol Ca^{++}/mg protein in the absence of oxalate, compared to a maximum of 240 nmol Ca^{++}/mg protein for vesicles from rabbit white muscle.

Table 1. Ca^{++} Accumulation by Sarcoplasmic Reticulum Vesicles from Red, White and Cardiac Muscle[1]

Source	Maximum amount of Ca^{++} uptake (μmol/mg protein)		Rate of Ca^{++} uptake (μmol/mg protein/min)		Reference
	with oxalate	without oxalate	with oxalate	without oxalate	
Heart, rabbit	.039	1.83	.256	---	Harigaya and Schwartz (1969)
Heart, dog	.075	3.00	---	---	Harigaya and Schwartz (1969)
Heart, human (failure)	.045	2.00	.034	---	Harigaya and Schwartz (1969)
Heart, dog	.026	2.32	---	.06	Katz and Repke (1967)
Skeletal, rabbit, white	.170	4.80	1.44	---	Harigaya and Schwartz (1969)
Skeletal, rabbit, red	.058	2.00	.182	---	Harigaya and Schwartz (1969)
Skeletal, rabbit, white	.240	6.00	1.80	1.8	Sreter (1969)
Skeletal, rabbit, red	.040	.3-1.1	.16	.16	Sreter (1969)
Skeletal, human, white	---	2.04	---	.38	Samaha and Gergely (1965a)
Skeletal, human, red	---	1.01	---	.17	Samaha and Gergely (1965b)

[1]Taken from Martonosi (1972)

III. MITOCHONDRIA

Massive Loading of Ca^{++}

Slater and Cleland (1953) found that heart mitochondria could absorb large amounts of Ca^{++}, but considered this to be a passive process. They also determined that carefully isolated mitochondria contained 10 mmol Ca^{++}/mg protein. DeLuca and Engstrom (1961) and Vasington and Murphy (1962) first discovered that isolated kidney mitochondria could accumulate up to 2.6 µmol Ca^{++}/mg protein in a respiration-linked process. Vasington and Murphy (1962) used rat kidney mitochondria suspended in an aerobic medium containing 10 mM respiratory substrate, 10 mM Mg^{++}, 3 mM ATP and 4 mM P_i, which was later termed "massive loading" conditions by Lehninger et al. (1967). Vasington and Murphy (1962) established that Ca^{++} uptake requires electron transport, plus the presence of ATP or ADP, Mg^{++}, and P_i, and that the mitochondria are uncoupled, so that no oxidative phosphorylation occurs. Ca^{++} uptake was inhibited by uncoupling agents, such as 2,4-dinitrophenol, but not by inhibitors of oxidative phosphorylation, such as oligomycin. Vasington and Murphy (1962) also observed that Ca^{++} uptake was completed after 10 minutes at 37°C. Lehninger et al. (1963) found that P_i was also accumulated under these conditions, and that calcium hydroxyapatite crystals were formed in the mitochondria, causing irreversible damage. Rossi and Lehninger (1963) found that Ca^{++} and P_i accumulation could be supported by

hydrolysis of ATP in the absence of electron transport, but accumulation under these conditions was inhibited by oligomycin.

Limited Loading of Ca^{++}

Under limited loading conditions, Lehninger et al. (1967) showed that the mitochondria are exposed to small concentrations of Ca^{++} (0.1-0.2 mM) and a low ratio of Ca^{++} to mitochondrial protein (100 nmol Ca^{++}/mg protein, or less). They concluded that after accumulation of one or more additions of Ca^{++}, rat liver mitochondria suffer no loss of respiratory control or capacity to phosphorylate ADP. Chance (1964) first observed that small amounts of Ca^{++} stimulated mitochondrial respiration. Drahota et al. (1965) established the fact that mitochondrial respiration returned to the resting rate when the Ca^{++} concentration fell to 1-2 μM. Chance (1964) demonstrated that even in the absence of ATP or P_i, mitochondria could accumulate Ca^{++}, but only to a maximum of 100 nmol Ca^{++}/mg protein. Rossi and Lehninger (1964) found that rat liver mitochondria accumulate Ca^{++} at concentrations down to 1.0 μM in preference to phosphorylating ADP. Rossi and Lehninger (1964) further demonstrated that in the presence of 2.0 mM inorganic phosphate, respiratory stimulation by Ca^{++} was prolonged and less than 10% of the Ca^{++} was retained by the mitochondria. They showed that addition of ATP and Mg^{++} restored the cyclic nature of respiratory stimulation by Ca^{++} and prevented the

discharging effect of excess phosphate.

Rasmussen et al. (1965) first observed that permeant anions, such as acetate, propionate or arsenate could replace phosphate and passively enter the mitochondria along with Ca^{++}, whereas, chloride was found to be an impermeant anion. When acetate was the permeant anion, the mitochondria swelled to the point of lysis during a Ca^{++}-induced respiratory jump, due to the accumulation of soluble calcium acetate, which increased the entry of water into the mitochondria (Rasmussen et al., 1965).

ATP-Supported Ca^{++} Accumulation

Under massive loading conditions, but in the absence of a respiratory substrate, Rossi and Lehninger (1963) found that 15 mM ATP readily supported Ca^{++} accumulation by rat liver and kidney mitochondria. However, 3 mM ATP did not support accumulation of Ca^{++} and P_i under similar conditions.

Using limited loading conditions, but also in the absence of a respiratory substrate, Bielawski and Lehninger (1966) found that addition of Ca^{++} to rat liver mitochondria in the presence of 0.5 mM ATP stimulated ATP hydrolysis for a short period, during which Ca^{++} was accumulated. Atractyloside, an inhibitor of mitochondrial uptake of ATP (Carafoli et al., 1965), and oligomycin inhibited ATP hydrolysis and Ca^{++} accumulation by mitochondria (Bielawski and Lehninger, 1966). Bielawski and Lehninger (1966) further demonstrated that for each ATP hydrolyzed, 2 molecules of Ca^{++} and

1 molecule of phosphate are accumulated. Brand and Lehninger (1977) observed similar stoichiometry for respiration linked Ca^{++} accumulation. They found that under limited loading conditions with or without phosphate, 2 moles of Ca^{++} were accumulated by the mitochondria for each pair of electrons passing each energy conserving site in the respiratory chain.

Proton Movement During Ca^{++} Accumulation

Using a glass electrode, Saris (1963) was the first to show that the suspending medium became more acidic during Ca^{++}-induced respiratory stimulation. Drahota et al. (1965) demonstrated that one H^+ atom was ejected for each atom of Ca^{++} accumulated, and that the exchange was reversible. The mitochondrial membrane is normally impermeable to H^+ (Mitchell and Moyle, 1965). Thus, ejection of H^+ during Ca^{++} accumulation is accompanied by a net alkalinization of the mitochondria, which can be measured by a titrimetric method for pH determination (Lehninger et al., 1967).

Comparison of Heart and Liver Mitochondria

Jacobus et al. (1975) found that rat liver mitochondria pulsed with 150nmol Ca^{++}/mg protein underwent a rapid reversible burst of respiration, and that Ca^{++} was accumulated in an energy linked process. Under similar conditions, they found that rat heart mitochondria exhibited only a marginal stimulation in the rate of oxygen consumption. Rat heart mitochondria retained the ability to phosphorylate ADP

in the presence of Ca^{++}, but in rat liver mitochondria addition of Ca^{++} completely uncoupled oxidative phosphorylation (Jacobus et al., 1975). Jacobus et al. (1975) also observed that 3.0 mM Mg^{++} inhibited Ca^{++} accumulation by rat heart mitochondria, but did not inhibit Ca^{++} accumulation by rat liver mitochondria. They then concluded that slower accumulation and release of Ca^{++} was characteristic of heart mitochondria.

In rat liver mitochondria, Mg^{++} did not stimulate oxygen consumption and was not accumulated under limited loading conditions (Lehninger et al., 1967). However, Brierley et al. (1963) found that Mg^{++} was accumulated by beef heart mitochondria if both a substrate and phosphate were present.

Sordahl (1974) found that in the presence of Ca^{++}, the addition of ADP to rabbit heart mitochondria stimulated respiration, but only if Mg^{++} was present. However, liver mitochondria under similar conditions were respiring maximally due to the presence of Ca^{++}, and addition of ADP did not increase respiration. Sordahl (1974) also observed that the presence of Mg^{++} markedly decreased the initial rate of Ca^{++} uptake by rabbit heart mitochondria, and also decreased the respiration rate. He then concluded that in rabbit heart mitochondria, Mg^{++} modulates the competitive effects of Ca^{++} and ADP for electron transport generated energy.

Mitochondrial Ca^{++} Release

Little is known about mitochondrial release of Ca^{++} in living muscle, and natural Ca^{++} releasing agents likely to operate *in vivo* have not been conclusively identified (Carafoli, 1976). Carafoli (1976) points out that mitochondrial Ca^{++} release must be studied in the absence of permeant anions such as phosphate, since the release of Ca^{++} stored in mitochondria as an insoluble salt is a slow process.

Haaker *et al.* (1972) demonstrated Ca^{++} accumulation is reversible by showing that in the absence of respiration, Ca^{++} efflux can be coupled to ATP synthesis. Under limited loading conditions, Carafoli *et al.* (1965) pointed out that Ca^{++} release may be measured by following the uptake of H^{+} from the medium, since Ca^{++} and H^{+} ion movement are reciprocal under these conditions.

Drahota *et al.* (1965) observed that rat liver mitochondria preloaded to a level of 57 nmol Ca^{++}/mg protein in the absence of P_i retained their Ca^{++} over a 20 minute period, if succinate was present. Mitochondria containing 143 nmol Ca^{++}/mg protein released 50% of their Ca^{++} during the same time period. Drahota *et al.* (1965) found that less Ca^{++} was released at higher mitochondrial concentrations. Below 0.5 mg of mitochondrial protein/ml, all the Ca^{++} was released (Drahota *et al.*, 1965). The spontaneous release of Ca^{++} could be prevented by addition of ATP and Mg^{++}, even if respiratory inhibitors such as cyanide, rotenone, or antimycin A were added. However, addition of either

2,4-dinitrophenol, oligomycin, or m-chlorophenylhydrazone caused Ca^{++} release (Drahota et al., 1965).

Drahota et al. (1965) further observed that rat liver mitochondria preloaded with Ca^{++} in the presence of ATP and P_i retained 98% of their Ca^{++} when centrifuged at 0^oC and resuspended in cold isotonic buffer, pH 7.4. Similar treatment at 30^oC resulted in the release of all the Ca^{++}. The Ca^{++} efflux was completely prevented by addition of respiratory substrates such as succinate (Drahota et al., 1965). Antimycin A prevented the sustaining action of succinate, indicating that resting respiration was necessary for Ca^{++} retention. Drahota et al. (1965) concluded that there was a continuous efflux of Ca^{++} from the mitochondria, balanced by a respiration-linked influx of Ca^{++}.

Sordahl (1974) found that the addition of electron transport inhibitors caused the release of 70% of the Ca^{++} accumulated by rabbit heart mitochondria in the presence of 8 mM P_i and 5 mM succinate. Allowing the mitochondrial suspension to become anaerobic also caused the release of 70% of the accumulated Ca^{++}. Addition of the ionophoretic antibiotic A-23187 to the mitochondrial suspension caused release of all accumulated Ca^{++}. Addition of ruthenium red, a specific inhibitor of mitochondrial Ca^{++} transport, caused efflux of 40-50% of the accumulated Ca^{++} (Sordahl, 1974).

Jacobus et al. (1975) found distinct differences in the rates of Ca^{++} efflux from heart and liver mitochondria. Ca^{++} uptake was much faster in liver mitochondria than in

heart mitochondria, but they also released Ca^{++} more rapidly. When 24nmol of Ca^{++} was added per mg protein, liver and heart preparations accumulated 25 and 12.6nmol Ca^{++}/mg protein in 10 seconds, respectively. After 3 minutes, however, the preparations contained 10 and 22.1nmol Ca^{++}/mg protein, respectively (Jacobus et al., 1975).

Kimura and Rasmussen (1977) demonstrated that depletion of the ATP levels of rat liver mitochondrial preparations to below 7μmol/mg protein resulted in the release of the accumulated Ca^{++}. Prostaglandins (Malstrom and Carafoli, 1975), phosphoenolpyruvate (Chudapongse, 1976) and Na^{+} (Carafoli, 1976) have been found to cause Ca^{++} efflux from mitochondria, but their significance as possible natural Ca^{++} releasing agents remains to be determined.

Identity of Mitochondrial Ca^{++} Binding Sites

Lehninger (1970) stated that mitochondria contained at least two sets of respiration-independent Ca^{++} binding sites. One has a low affinity for Ca^{++}(K_m=100 μM) but is more numerous, binding 40-60nmol Ca^{++}/mg protein. The other has a high affinity for Ca^{++}(K_m=0.5 μM), but is less numerous and binds only 1.0nmol Ca^{++}/mg protein. Lehninger (1970) further observed that low affinity binding of Ca^{++} was non-specific, binding to polar membrane proteins and lipids. The high affinity binding sites for Ca^{++} were present only in mitochondria of species capable of accumulating Ca^{++} during respiration. Blowfly muscle mitochondria were not capable of

respiration-linked Ca^{++} accumulation, and contained no high affinity Ca^{++} binding sites (Lehninger, 1970). Mitochondria that contained high affinity Ca^{++} binding sites were not dependent on respiration or the presence of ATP to bind Ca^{++} (Lehninger, 1970).

Knowing that ruthenium red is a specific inhibitor of Ca^{++} uptake by mitochondria and is also a dye specific for glycoproteins, Carafoli (1975) attempted to locate the Ca^{++} binding components in the glycoprotein fraction of mitochondria. Carafoli (1975) found that the Ca^{++} binding glycoprotein had a molecular weight of 33,000 and contained 10% carbohydrate, which included one sialic acid residue per mole. The glycoprotein was very anionic, contained a variable amount of phospholipid and comprised about 1% of the total liver mitochondrial protein. The Ca^{++} binding glycoprotein was found in the mitochondrial membrane and in the intermembrane space (Carafoli, 1975).

Reed and Bygrave (1975) determined by kinetic analysis of Ca^{++} transport that the binding site of the mitochondrial Ca^{++} carrier contained 3 carboxylate residues in close proximity to a tertiary nitrogen. It had a pK_a near 7.0, which is similar to histidine.

Ca^{++} Transport and the Mechanism of Oxidative Phosphorylation

The recently proposed paired moving charge theory of mitochondrial oxidative phosphorylation (Blondin and Green, 1975) provides the most likely explanation of the mechanism

of mitochondrial Ca^{++} accumulation. Currently, the most widely accepted theory for the mechanism of oxidative phosphorylation is either the chemical-coupling, the conformational-coupling, or the chemiosmotic-coupling hypothesis (Lehninger, 1975). Blondin and Green (1975) dismissed the chemical-coupling hypothesis because the proposed high energy intermediates necessary for ATP synthesis or ion transport have never been isolated. With regard to the conformational-coupling hypothesis (Lumry, 1974), Blondin and Green (1975) indicated that there may be a conformational component in bond rupture and formation, but that is the extent to which conformational energy could play a role in energy coupling. The chemiosmotic-coupling hypothesis proposes that the proton gradient generated by electron transport would drive the mitochondrial ATPase reaction in the reverse direction, thereby forming ATP. Thayer and Hinkle (1973) found that an imposed pH gradient can drive ATP synthesis in mitochondria, but Blondin and Green (1975) concluded that this observation only proves that the mitochondrial ATPase reaction is reversible. Blondin and Green (1975) further point out that nonmembraneous submitochondrial particles retain energy coupling functions. However, the chemiosmotic-coupling hypothesis predicts that energy coupling requires a membrane with a transmembrane potential.

In the paired moving charge hypothesis, the electron is a charged moving ion as it passes down the electrochemical potential in its transit through the electron transfer chain

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(Blondin and Green, 1975). The moving negative charge has a coulombic attraction for cations that is effective over distances of 20 to 30 $\mathring{A}$, and is probably the criterion responsible for the characteristic structure of the mitochondrial cristae (Blondin and Green, 1975). This model proposes that mitochondrial ATP synthetase contains an ionophore-protein that separates ADP and P_i anions from their cations, leaving the cations in the aqueous media. Blondin and Green (1975) proposed that the ADP anion forms a positively charged ionophore complex with Mg^{++}. They also proposed the existence of a track protein that provides a transmembrane pathway for the ionophore complex. According to this theory, an acceptor protein containing bound AMP is the acceptor for the positively charged P_i-ionophore complex, forming bound ADP. The bound ADP then acts as the phosphate donor for the positively charged ADP-MG^{++}-ionophore complex. The vectorial arrangement of the electron transport chain drives the reacting complexes together due to the synchronous movement of the positively charged ionophore complex and the electron across the membrane (Blondin and Green, 1975). Similar to the chemiosmotic hypothesis protons are ejected into the medium (Lehninger, 1970). Blondin and Green (1975) proposed a similar ionophore-mediated process for cation transport, and identified 13-hydroxy-octadecadienoic acid as the ionophore for Na^+.

Blondin and Green (1975) proposed that mitochondrial uncouplers serve as substitutes for ADP or P_i in the two

ionophore complexes. They also act as proton carriers and as membrane soluble anions. They stated that in each coupling cycle, 2 uncoupler molecules are required. One molecule moves cyclically, electrostatically linked to the ionophore-encapsulated Mg^{++}. The other molecule alternates from the protonated form, when moving in the same direction as the electron, to the anionic form, when moving in the opposite direction (Blondin and Green, 1975). Blondin and Green (1975) suggested that the uncouplers are cycled back and forth, with no net chemical work done, resulting in the supression of ATP synthesis without depressing electron flow. They concluded that uncouplers are actually "super-couplers".

Inhibitors of oxidative phosphorylation suppress both oxidative phosphorylation and electron flow in mitochondria (Lehninger, 1975). According to the model proposed by Blondin and Green (1975), such reagents substitute for ADP or P_i, inhibiting ATP synthesis. These reagents may not be protonated under experimental conditions. They concluded that electron flow is inhibited because of the lack of a paired moving charge for the electron.

Regulation of Ca^{++} Movements by Mitochondria

Huddart and Price (1976) pointed out that any intracellular organelle capable of controlling contraction must be able to cyclically raise and lower the cellular free Ca^{++} concentration over a range of 10^{-7} to 10^{-5} M in 2 to 10 milliseconds. They stated that the muscle sarcoplasmic

reticulum possesses these properties, but mitochondria do not. Huddart and Synson (1975) suggested that muscle mitochondria may modulate Ca^{++} levels during contraction due to the greater abundance of mitochondrial membrane as compared to sarcoplasmic reticulum.

Carafoli (1975) observed that one-half of the 150 nmol Ca^{++}/mg mitochondrial protein is bound to energy independent sites. Carafoli (1975) further observed that heart muscle contain 80 mg mitochondrial protein/g tissue. He calculated that during one heartbeat, 25 nmol Ca^{++}/g tissue is exchanged between the myofibrils and the sarcoplasm. He concluded that only 0.3 nmol Ca^{++} is transported/mg protein/heart beat. Carafoli (1975) suggested that Ca^{++} binding may occur at mitochondrial energy independent sites. No changes in the parameters of respiration dependent Ca^{++} binding would be observed under these circumstances (Carafoli, 1975).

Intracellular Localization of Ca^{++}

Popescu et al. (1975) reported that the highest concentration of Ca^{++} was found in the nuclear fraction, followed by the mitochondrial, SR, and sarcolemmal fractions of guinea pig smooth muscle in order of decreasing Ca^{++} concentrations. The sarcoplasmic reticulum contained only 25% of the total mitochondrial Ca^{++}, but this amount was sufficient to cause muscle contraction (Popescu et al., 1975). Winegrad (1965) observed by autoradiography that the Ca^{++} in frog toe muscles was found primarily in the sarcoplasmic

reticular terminal cisternae and in the areas of the actin/myosin interaction. Legato and Langer (1969) found that in dog papillary muscles, Ca^{++} was located primarily in the lateral sacs of the SR and in the I-band of the sarcomere.

Slater and Cleland (1953) reported that all of the Ca^{++} of rat heart muscle (1 µg Ca^{++}/g heart) was recovered in the isolated mitochondria. Patriarca and Carafoli (1968) showed that after intraperitoneal injection of $^{45}CaCl_2$ into rats, most of the label was associated with the isolated mitochondrial fraction. Martonosi (1972) explained that tissue homogenization and fractionation disrupted the SR to a greater extent than in mitochondria, so that the mitochondria retained a greater ability to accumulate Ca^{++}. Martonosi (1972) concluded that the Ca^{++} may be redistributed during tissue homogenization and fractionation. Martonosi (1972) then concluded that localization of large amounts of Ca^{++} in the mitochondria of living muscle is improbable.

MATERIALS AND METHODS

Isolation of Sarcoplasmic Reticulum

Beef Sternomandibularis (neck) or rabbit Longissimus dorsi (back) muscles were excised immediately after slaughter, trimmed to remove excess fat and connective tissue, and either ground in a chilled meat grinder or finely diced with a razor blade. All subsequent steps (Fig. 3) were conducted at $0^{o}C$, using the procedure of Meissner and Fleisher (1971).

The ground or diced muscle (150-200 g) was suspended in 2 volumes (400 ml) of homogenization buffer and homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, N.Y.) in 50 ml aliquots for 30 seconds at a setting of 0.5. The homogenization buffer consisted of 0.3 M sucrose and 0.01 M HEPES (N-2-hydroxyethylpiperazine-n'-1-ethansulfonic acid), pH 7.4.

The muscle homogenate was centrifuged for 20 minutes at 8000 g (7000 rpm) using a Sorvall centrifuge (Model RC2-B, Sorvall, Inc.) with a GSA rotor, to remove the myofibrillar and mitochondrial fractions. The supernatant was strained through 8 layers of cheesecloth to remove floating fat particles. A crude fraction of sarcoplasmic reticulum vesicles was obtained by centrifugation for 75 minutes at

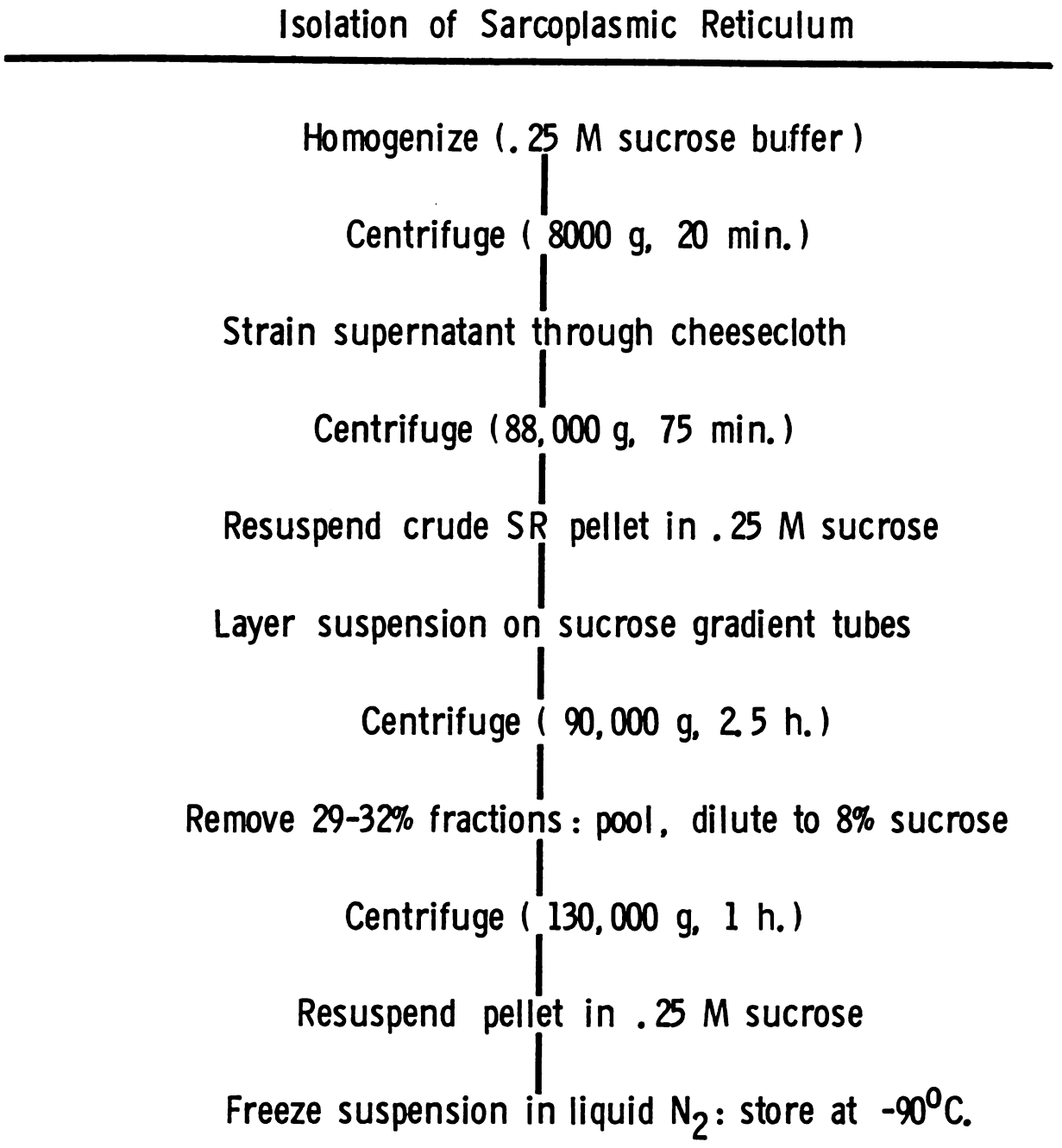

Fig. 3. Flow sheet of procedure used in the isolation of sarcoplasmic reticulum vesicles.

88,000 g (28,000 rpm) using an IEC model B-60 preparative ultracentrifuge (International Equipment Company, Needham Heights, Mass.) equipped with an A-170 rotor. The pellets were resuspended and pooled in 18 ml of the homogenization buffer, using a Pasteur pipette. The pooled suspension was homogenized briefly using a chilled Polytron homogenizer.

Three ml of crude sarcoplasmic reticulum suspension were then layered on six tubes containing a discontinuous sucrose gradient buffered with 5 mM HEPES, pH 7.4. The gradients were prepared as follows:

1) The polypropylene centrifuge tubes were filled with a solution of 5% $K_2Cr_2O_7$ in either 50% H_2SO_4 or 70% HNO_3 for 36 hours at room temperature. The tubes were thoroughly washed and were then permanently wettable (Wallace, 1969).
2) Solutions of 26.4, 29.1, 31.6, 33.9 and 37.2% sucrose (w/w) were prepared by weighing out 29.3, 32.7, 35.9, and 43.2 g of sucrose, respectively, adding 5 ml of 100 mM HEPES, pH 7.4, and bringing the volume to 100 ml. These solutions could be stored frozen for later use.
3) Using a Pasteur pipette, 2.2 ml of the 37.2 sucrose solution was placed in the bottom of each centrifuge tube. Then 2.0 ml of the 33.9% solution was carefully layered on top of the 37.2% solution, followed by 1.6 ml of the 31.6% solution, 1.6 ml of the 29.1% solution, and 2.4 ml of the 26.4% solution.

The centrifuge tubes, with 3 ml of the suspension layered on each, were placed in an SB-283 rotor of the IEC preparative ultracentrifuge and centrifuged for 2.5 hours at 90,000 g (23,500 rpm).

After centrifugation, the top 4.8 ml in each tube was carefully removed and discarded. Using a Pasteur pipette, the next 3.9 ml in each tube (including all of the 29.1 and 31.6% sucrose layers, and a portion of the 26.4 and 33.9% layers) were removed and pooled. This fraction was diluted with 2 volumes (47 ml) of 5 mM HEPES, pH 7.4, which was added over a 30 minute period to minimize osmotic shock. The diluted suspension was then centrifuged for 1 hour at 130,000 g (35,000 rpm) using the A-170 rotor of the IEC preparative ultracentrifuge.

The sarcoplasmic reticulum pellets were resuspended and pooled in 2-5 ml of 0.3 M sucrose and 2.5 mM HEPES, pH 7.4. Protein concentration was determined before freezing, using the method of Lowry et al. (1951). The resuspended vesicles can be stored overnight at $0^{o}C$ without loss of Ca^{++} accumulating ability (Kanda, 1975), or frozen in liquid nitrogen and stored at $-70^{o}C$ for several months (Meissner, 1974). Ultrapure sucrose was used for all solutions.

Isolation of Mitochondria

Mitochondrial fractions were isolated (Fig. 4) from the beef Sternomandibularis and the rabbit Longissimus dorsi by differential centrifugation using the procedure of Ernster and Nordenbrand (1967). The excised muscle was trimmed to remove excess fat and connective tissue, weighed, and immersed in ice cold 0.15 M KCl. The remaining steps were done at 0^{o}C. The tissue was minced and rinsed with several changes of 0.15 M KCl. The minced tissue was rinsed with Chappell-Perry medium, and then suspended in 1 volume of Chappell-Perry medium, which consists of 0.1 M KCl, 0.05 M Tris-HCl buffer, pH 7.4, 0.001 M Na-ATP, 0.005 M $MgSO_4$, and 0.001 M EDTA (ethylenediaminetetracetate).

The suspension was homogenized for 20-30 seconds using a Polytron homogenizer at a setting of 0.5. The homogenate was diluted with Chappell-Perry medium to a volume of 10 times the initial weight of the muscle and centrifuged with a Sorvall RC2-B refrigerated centrifuge at 650 g (2000 rpm) for 10 minutes to remove the myofibrillar fraction. The supernatant was centrifuged as before to remove residual myofibrils. The supernatant was decanted and centrifuged at 10,000 g (8,000 rpm) for 10 minutes. The mitochondrial pellet was resuspended in Chappell-Perry medium and recentrifuged as before. The tightly packed mitochondrial pellet was rinsed with 0.25 M sucrose to remove the remainder of the medium, and then suspended in 2-6 ml of 0.25 M sucrose and 10 mM HEPES buffer, pH 7.4, to contain 6-10 mg protein/ml.

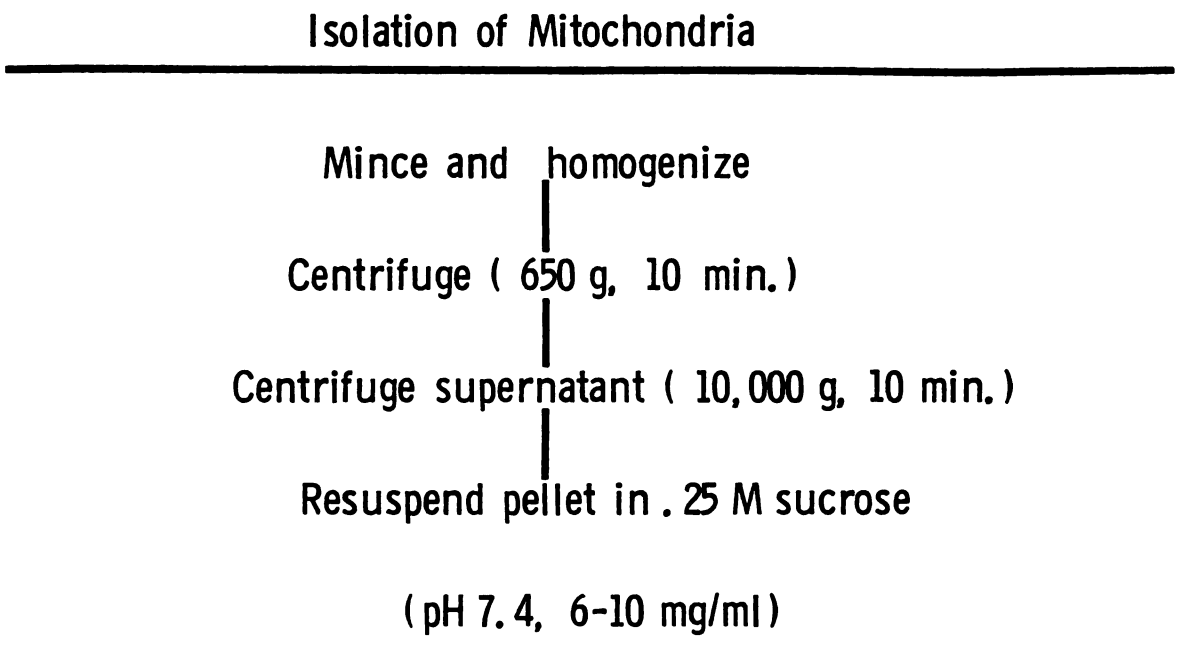

Fig. 4. Flow sheet of procedure used in the isolation of mitochondria.

Protein concentration was measured by the method of Lowry et al. (1951). Mitochondrial suspensions were stored in ice ($0^{o}C$) and all experiments were completed by 24 hours after isolation.

Protein Determination

The protein concentration was determined using the method of Lowry et al. (1951). Lowry solution A (20 g Na_2CO_3, 4 g NaOH and 0.2 g $KNaC_4H_4O_6 \cdot 5H_2O$/1) was mixed in the ratio of 50:1 with Lowry solution B (6 g $CuSO_4 \cdot 5H_2O$/L) immediately prior to use to give Lowry solution C. Phenol solution was prepared immediately prior to use by diluting Folin and Ciocalteu phenol reagent (Harleco, Philadelphia, Penn.) 1:1 with distilled water.

To assay for protein 5 ml of Lowry solution C was added to 1 ml of appropriately diluted protein solution, and the mixture was incubated for 20 minutes at room temperature. Then 0.5 ml of the diluted phenol solution was rapidly added and mixed. The mixture was allowed to stand with occasional shaking for 45 minutes at room temperature for color development. Absorbance was measured at 660 nm against a control consisting of water plus all other reagents. The protein concentration was determined by comparison with a standard curve prepared from crystalline bovine serum albumin (Fig. 5).

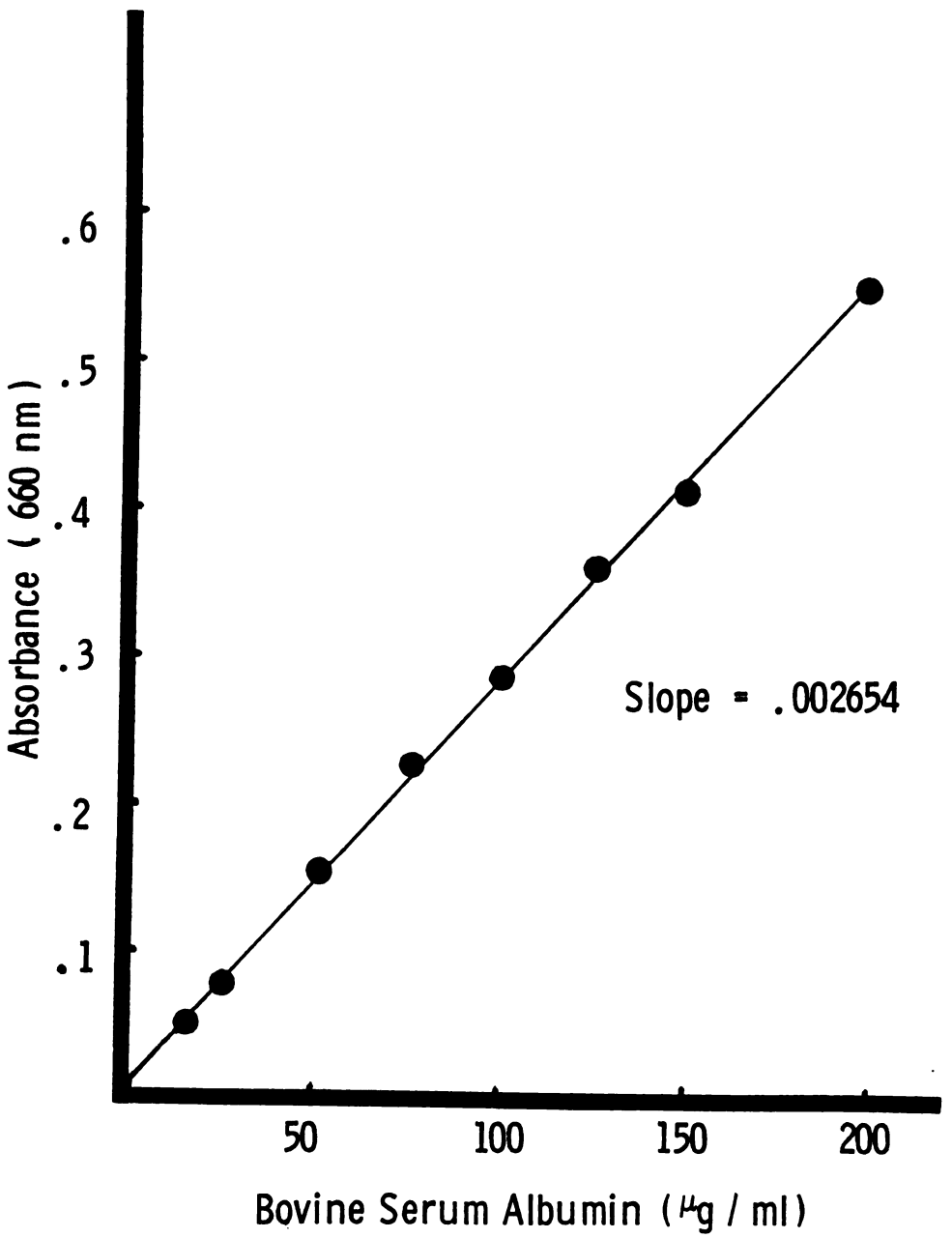

Fig. 5. Standard curve for the determination of protein by the method of Lowry et al. (1951).

A Manometric Assay for Mitochondrial Succinate Oxidase

Mitochondrial succinate oxidase activity was determined manometrically, using a Gilson Respirometer (King, 1967). The following reagents were prepared:

Phosphate buffer, 0.2 M (the Sorenson type), pH 7.8

Succinic acid, 0.6 M in water, adjusted to pH 7.8 with NaOH

Cytochrome c, 0.0009 M in water

In the main compartment of a Warburg flask were placed 0.1 ml cytochrome c, 1.5 ml phosphate buffer, and an amount of the preparation to give 10-80 µL of oxygen uptake per 10 minutes. Water was added up to 2.8 ml. In the side arm, 0.2 ml succinate was pipetted.

Three or four levels of the preparation were tested. Diffusion may become limiting when the reaction is fast. After temperature equilibration at 30°C for 8 minutes, the succinate was tipped into the main compartment. Readings were taken for 1 hour at 10-minute intervals. The oxygen uptake was linear with time for the first 30 minutes. Oxygen uptake was corrected to STP (standard temperature and pressure). Oxygen uptake (µL) was converted to mM succinate oxidized by multiplying by 0.0298 (King, 1967). The specific activity of succinate oxidase was expressed as µmol succinate oxidized/min/mg protein (King, 1967).

Measurement of Mitochondrial Respiration

Mitochondrial protein (3-5 mg) in 0.5 ml of 0.25 M sucrose was added to a Warburg vessel which contained 1.5 ml of a medium consisting of 50 mM KCl, 25 mM Tris-HCl buffer, pH 7.5, 25 mM K-phosphate buffer, pH 7.5, 0.2% bovine serum albumin, 4-8 mM $MgCl_2$, 10 mM substrate (succinate), 1 mM ATP, 30 mM glucose, 100 Kunitz-MacDonald units of hexokinase and 0.01-0.02 mM cytochrome c. After temperature equilibration at 30°C for 5 minutes, manometric readings were taken at 5 minute intervals for 30-40 minutes. Oxygen uptake (µL) was converted to mM oxygen consumed by using a factor of 0.0149 (King, 1967). Respiration was expressed as µmol O_2/mg protein/minute (King, 1967).

Determination of Ca^{++} Accumulation in Sarcoplasmic Reticulum Vesicles

The Ca^{++} transport system of sarcoplasmic reticulum requires ATP, Mg^{++} and Ca^{++} for activation. Thus, the reaction mixture contained 100 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM $^{45}CaCl_2$ and 10 mM histidine, pH 7.3. The $^{45}CaCl_2$ was obtained in aqueous solution (Amersham-Searle Corp., Arlington Heights, Illinois). Sufficient $^{45}CaCl_2$ was added to a 5 ml stock solution of 10 mM $CaCl_2$ to give a final count of 20,000 cpm per ml of reaction mixture.

The reaction mixture, without $CaCl_2$, was adjusted to the desired pH (7.3, 6.8, 6.2, 5.5 or 5.0) with 1.0 N HCl or 1.0 N KOH, as needed. An appropriate volume of the reaction

mixture (usually 4 ml) was placed in a tube and equilibrated to 38, 15 or 0°C. Ten µL of the 10 mM $^{45}CaCl_2$ stock solution was added per ml reaction mixture, so that the reaction mixture contained 100 nmol $^{45}Ca^{++}$/ml, and approximately 20,000 cpm/ml.

The Ca^{++} accumulation reaction was initiated by addition of 40-300 µg of sarcoplasmic reticulum protein per ml of reaction mixture. Aliquots of 0.5 ml were removed at various intervals (1,2,4,8,13 and 18 minutes) and the reaction was terminated by filtration through a Millipore filter, type GS, with an average size of 0.22 µM (Martonosi and Feretos, 1964). The filtrate was collected in a test tube, and the radioactivity in the filtrate was determined by liquid scintillation counting. The Ca^{++} accumulated was determined by the following equation (see Fig. 6):

$$A = \frac{B \times \frac{C - D}{C}}{E}$$

A = Ca^{++} accumulated in nmol/mg

B = Ca^{++} concentration in reaction medium expressed as nmol/ml

C = Control cpm (counts per minute)

D = Sample cpm

E = Protein concentration of sample (mg/ml)

Determination of Ca^{++} Accumulation by Mitochondria

Ca^{++} accumulation was determined under "limiting loading" conditions (Lehninger et al., 1967). The reaction mixture contained 10 mM succinate, 3 mM ATP, 210 mM mannitol,

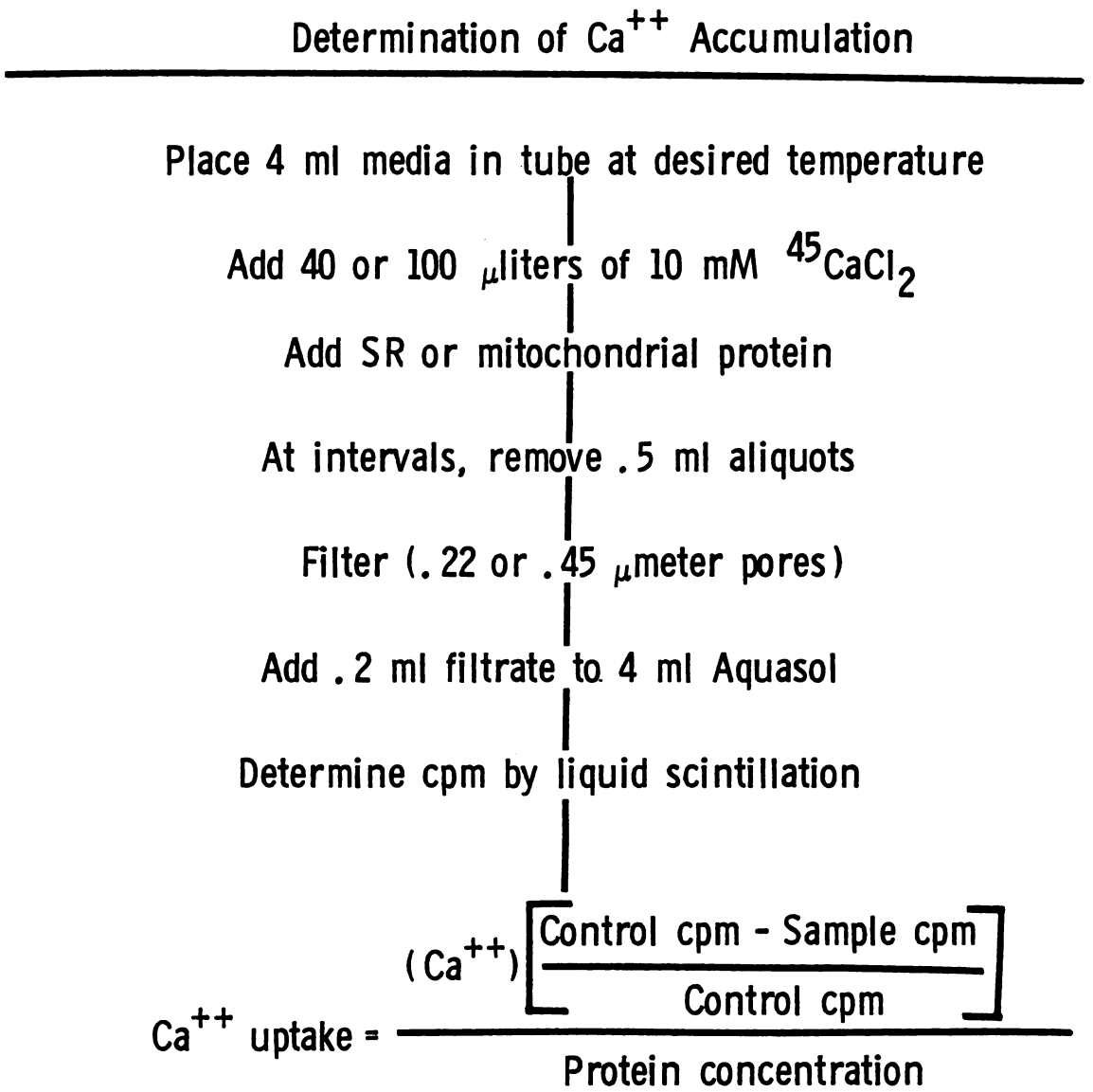

Fig. 6. Flow sheet of the procedure used for the determination of Ca^{++} accumulation.

70 mM sucrose and 10 mM Tris buffer, pH 7.4 (Jacobus et al., 1975; Carafoli, 1976). A stock solution of 10 mM $^{45}CaCl_2$ was prepared as described previously.

After adjusting the pH, 4 ml of the reaction mixture was placed in a tube and equilibrated to 38, 15 or 0°C. Twenty five µliters of the $^{45}CaCl_2$ solution was added per ml reaction mixture, so that the reaction mixture contained 250nmol $^{45}Ca^{++}$/ml, and approximately 20,000 cpm/ml.

The reaction was initiated by addition of 0.5-0.8 mg protein/ml reaction mixture. The reaction was terminated at various time intervals by filtration through a Millipore or Gelman filter with an average pore size of 0.45 µM (Jacobus et al., 1975). Ca^{++} accumulation was calculated as previously described (Fig. 6).

Determination of Ca^{++} Release in Sarcoplasmic Reticulum Vesicles or Mitochondria

Ca^{++} accumulation was allowed to proceed for 8 minutes. A portion of the mixture was then transferred to another tube. Calcium accumulation was measured in both tubes at 13 and 18 minutes. One tube served as a control. The second tube was subjected to any of several different conditions. Some tubes were placed in another water bath at the desired temperature. In some experiments, the pH of the mixture was lowered to pH 5.0 by addition of 0.1 N HCl, using a Corning model 12 pH meter to monitor pH. Nitrogen gas was bubbled through some tubes, using a disposable pipette as the hose

tip. Ca^{++} release was determined by comparison of the Ca^{++} accumulated at 8 minutes with the Ca^{++} accumulated under the changed conditions at 13 and 18 minutes.

Determination of Radioactivity

Aliquots of the filtrate (0.2 ml) were mixed with 4 ml of Aquasol-2, a scintillation liquid (New England Nuclear, Boston, Mass.). Sample radioactivity was determined using a Beckman liquid scintillation counter, model 3133P, using Channel B set at 1.5% preset error.

SDS Gel Electrophoresis of Beef and Rabbit Sarcoplasmic Reticulum

Sodium dodecyl sulfate (SDS) electrophoresis was carried out using the method of Weber and Osborn (1969) as modified by Porzio and Pearson (1976). Sarcoplasmic reticulum pellets were dissolved in a solution composed of 1% SDS, 5 mM ethylenediaminetetraacetate (EDTA), 1 mM dithiothreitol and 50 mM Tris/glycine, pH 8.8. A stock solution of 2.0 M Tris/glycine (0.5 M Tris:1.5 M glycine, pH 8.8) was previously prepared. Samples were heated for 5 minutes at 100°C to aid in dissolving the proteins, then dialyzed overnight against 25 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol and 0.2% SDS. Glycerol was added to a concentration of about 20%, and several drops of Pyronin Y tracking dye (0.25 mg/ml) were added to the sample immediately before loading onto the gel.

For the 10% acrylamide (100:1) gel system, a stock solution was prepared by dissolving 25.0 g acrylamide and 0.25 g bis in deionized water and bringing the final volume

to 100 ml. The solution was then passed through a 2 x 4 cm column of dry packed mixed bed deionizing resin before use. Stock solutions of 2.0 M Tris/glycine, 50% glycerol, 2.5% SDS/2.5 mM EDTA, 1% N, N, N', N'-tetramethylethylene-diamine (TEMED), and 1% ammonium persulfate were prepared. The casting solution consisted of stock solutions in the following proportions: 10 volumes acrylamide, 5 volumes Tris buffer, 2.5 volumes glycerol solution, 1 volume SDS/EDTA, 4.5 volumes water and 1 volume TEMED. The casting solution was deairated by application of vacuum, and 1 volume of the initiator, ammonium persulfate, was added immediately before casting. After the gels were loaded to within 5 mm of the top of the tube (8 cm x 5 mm ID tubes), a layering solution consisting of 400 mM Tris/glycine (pH 8.8), 0.04% TEMED, 0.1% SDS and 0.004% ammonium persulfate was layered on top of the acrylamide solution to form a smooth interface. After polymerization was completed (20 minutes), the layering solution was removed and the gel surface was relayered with a solution consisting of 400 mM Tris/glycine (pH 8.8), 5% glycerol and 0.1% SDS for an hour before use.

Electrophoresis was conducted in a Buchler Polyanalyst unit. Each chamber contained 340 ml of chamber buffer consisting of 200 mM Tris/glycine (pH 8.8) and 0.1% SDS. A Heathkit IP-17 constant voltage power supply of 0-400 V, 100 mA capacity was employed. Protein samples (25-50 μL) were loaded on the gel and entry of the sample into the gel initiated at 0.5 mA per gel. After the dye had completely

entered the gel, the current was raised to 1.0 mA per tube (12 mA at 40 V for 12 gels). Migration continued until the dye front was within 5 mm of the bottom of the gel. The gels were removed from the tubes and the dye front was marked by insertion of a fine wire.

The gels were fixed by soaking in a solution of 25% (v/v) isopropanol/10% (v/v) acetic acid for several hours. The samples were stained overnight in a solution consisting of 0.01% Coomassie Brilliant Blue R 250, 50% (v/v) methanol, and 7.5% (v/v) glacial acetic acid. The gels were destained in 10% (v/v) acetic acid/5% (v/v) methanol.

A standard curve for molecular weight determination was prepared using the following proteins: myosin - 200,000; γ-globulin (unreduced) - 160,000; muscle C-protein - 140,000; β-galactosidase - 130,000; α-actinin - 102,000; bovine serum albumin - 68,000; catalase - 60,000; ovalalbumin - 43,000; glyceraldehyde dehydrogenase - 36,000; myokinase 21,500; haemoglobin - 15,500 (Fig. 7).

Transmission Electron Microscopy

Small muscle samples, sarcoplasmic reticulum vesicles, or mitochondria obtained by centrifugation were fixed using a modification of the procedure described by Sjostrand (1967). Samples were fixed for 2 hours in a buffer solution containing 1.25% glutaraldehyde, 0.048 M sodium phosphate (pH 7.4) and 0.043 M NaCl (415 milliosmolar solution). The tissue samples were washed for 1 hour in 2 changes of

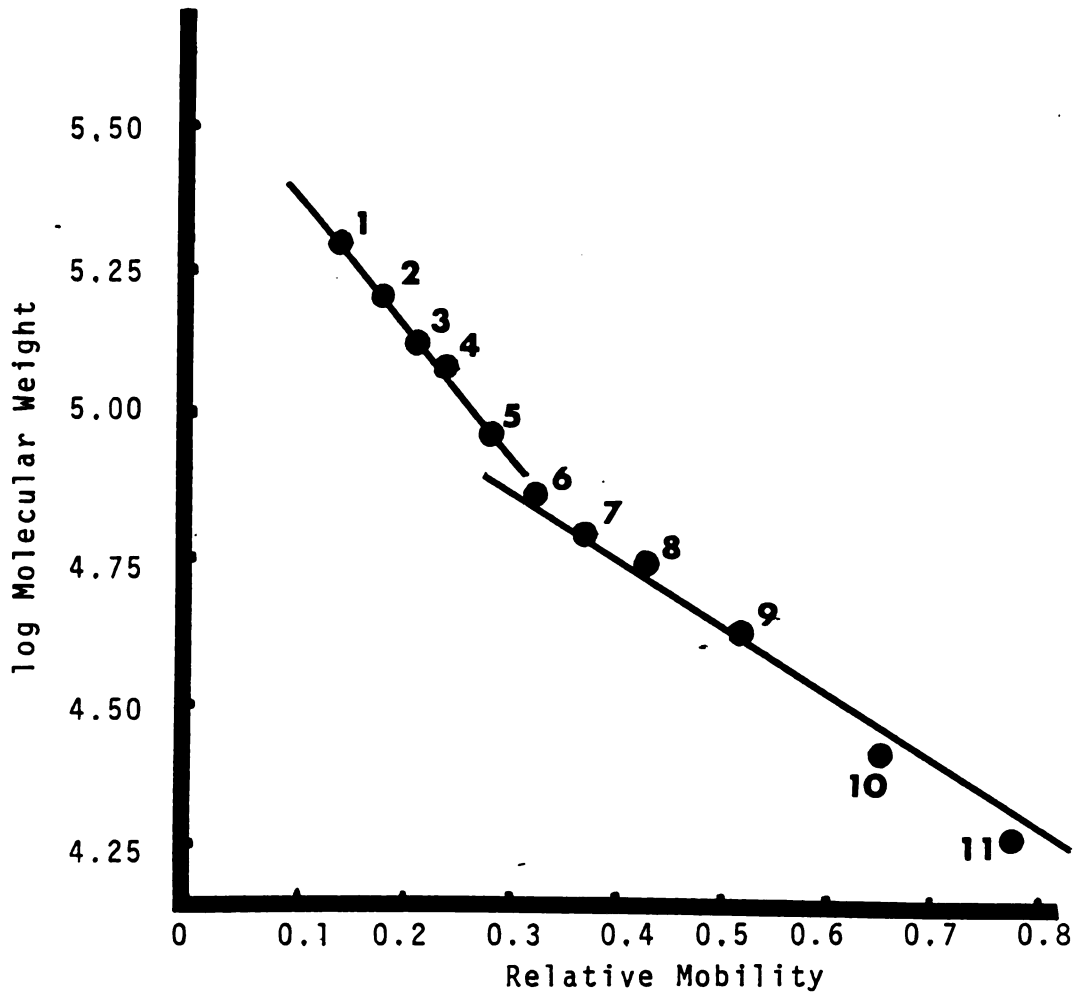

Fig. 7. Standard plot of log molecular weight and relative mobility on 10% acrylamide SDS-gel with 0.10% crosslinker. The protein standards and their molecular weights are as follows: (1) myosin - 200,000; (2) γ-globulin (unreduced) - 160,000; (3) muscle C-protein - 140,000; (4) β-galactosidase - 130,000; (5) α-actinin - 102,000; (6) bovine serum albumin - 68,000; (7) catalase - 60,000; (8) ovalalbumin - 43,000; (9) glyceraldehyde dehydrogenase - 36,000; (10) myokinase - 21,500; (11) haemoglobin - 15,500.

0.094 M sodium phosphate and 0.043 M NaCl buffer solution at pH 7.4. The samples were then postfixed for 1 hour in 1% osmium tetroxide solution in veronal acetate buffer (pH 7.4), which was adjusted to 300 milliosmolar with NaCl, KCl, and $CaCl_2$. The composition of the buffer solutions and the fixative preparation schedules are shown in Appendix Table 1 - 6.

After fixation, the samples were dehydrated for 15 minutes each in 25, 50, 75, and 95% ethanol. They were then placed in 2 changes of 100% ethanol for 30 minutes each. The dehydrated samples were transferred through 2 changes of propylene oxide for 30 minutes each, followed by 12 hours in a 1:1 mixture of propylene oxide and epon-araldite resin. The samples were embedded in pure epon-araldite resin using flat embedding molds (LKB Instruments, Inc.). The embedded samples were placed in a desiccator under slight vacuum for 12 hours, then placed in a $80^{o}C$ oven for 36 hours to allow the blocks to harden.

Epon-araldite embedded tissue blocks were trimmed by hand with a razor blade, and sectioned with either a diamond or glass knife to a thickness of 60 to 100 nM using an LKB 4801 ultramicrotome. Sections were picked up from the knife boat on uncoated 300 mesh copper grids. Staining of the tissue was accomplished by floating the grids for 30 minutes on a saturated solution of uranyl acetate, followed by thoroughly rinsing with distilled water, and then staining for 5 minutes in a solution of lead citrate (Abbott, 1976).

The sections were washed with 0.02 M NaOH, followed by distilled water, and air dried.

A Phillips EM-300 transmission electron microscope was used for observing the stained sections at an accelerating voltage of 60 KV. Photographs of each sample were taken using Kodak 8.25 x 10.16 cm sheet film. The film was developed for 4 minutes in Kodak D-19 developer, washed for 1.5 minutes in running water, fixed 8 to 10 minutes in Kodak fixer, washed in running water for 1 minute, rinsed in Kodak Hypo-Clearing Agent, and washed for 10 minutes. The washed negatives were dipped in Kodak Photo-Flo solution and dried for 45 minutes with warmed air. All of the previous steps were performed using an Arkay nitrogen burst machine.

Kodak Polycontrast Rapid Resin Coated Paper was exposed from the negatives using a Durst S-45-EM enlarger. The exposed paper was developed in Kodak Dektol developing solution (stock solution diluted 1:2 with water) for 1.5 minutes. The prints were rinsed in Kodak Indicator Stop Bath for 5 seconds, and fixed for 2 minutes in a solution of Kodak Fixer. The prints were then washed 4 minutes in running water. The surface of the prints was blotted with a soft sponge to remove water droplets, and air dried at room temperature. All solutions were at 18-21^{o}C during processing.

Muscle Fiber Type Determination

Immediately after slaughter muscle samples were removed and trimmed to small rectangular pieces approximately 3.5 x

3.5 x 5 mm, with the long (5 mm) dimension of the sample being cut parallel to the longitudinal direction of the muscle fibers. The samples were then wrapped in Saran wrap and aluminum foil, submerged in liquid nitrogen until frozen, and then placed in a previously chilled (-20^{o}C) Slee-Pearse cryostat. The frozen samples were positioned on a microtome chuck using O.C.T. compound (Miles Laboratories), so that cross sections of 10-12 μM in thickness could be obtained. The sections were placed on coverslips and allowed to dry at room temperature for 30 minutes.

Fiber type was determined by the reduced diphosphopyridine nucleotide-tetrazolium reductase method of Engel and Brooke (1965), which stains mitochondria. Red or oxidative fibers appear dark blue when viewed with a light microscope. White or glycolytic fibers remained unstained.

The following schedule was used for staining the tissue sections:

1. Using a magnetic stirring bar, 20 mg of Nitro-blue Tetrazolium Chloride was dissolved in 20 ml of 0.2 M Tris buffer, pH 7.4.
2. After mixing, 16 mg of NADH (reduced) was added and the cover slips with the adherring tissue sections were placed in the mixture.
3. The sections were incubated for 30 minutes at 36-38^{o}C in a shaker water bath.
4. The sections were post-fixed for 3 minutes in 10% formaldehyde solution.

5. The tissue was rinsed in 3 changes of distilled water.
6. The coverslips were mounted on glass slides using Permount (Fisher).

Photographs of the stained sections were taken on a Zeiss Photomicroscope III, using Kodak Panatomic-X film. The film was developed at 20°C in a small lightproof tank using Kodak Microdol-X developing solution for 7 minutes. The film was rinsed for 30 seconds in Kodak Indicator Stop Bath solution, fixed for 2-4 minutes in Kodak Fixer solution, washed for 20-30 minutes in running water (18-24°C), and dipped in Kodak Photo-Flo solution before drying at room temperature.

Enlargements were made using Kodak Polycontrast Resin Coated Paper as previously described.

RESULTS AND DISCUSSION

Cold Shortening in Beef and Rabbit Muscles

Figure 8 shows beef muscle which has been subjected to cold shortening. Chilling beef Sternomandibularis muscle to $0^{o}C$ resulted in 43% shortening, but at $15^{o}C$, very little shortening occurred. No appreciable shortening occurred in rabbit Longissimus dorsi muscles at either 15 or $0^{o}C$. These results are similar to those reported by Locker and Hagyard (1963), and are included only to illustrate the extent to which cold shortening occurs in red muscles.

Purity and Characterization of Sarcoplasmic Reticulum Preparations

Working in this laboratory, Kanda (1975) established that SR prepared by the procedure of Meissner and Fleisher (1971) was essentially free of contamination from lysosomes, mitochondria, and plasma membrane. This was verified by measuring acid phosphatase activity, succinate-cytochrome c reductase activity, and 5'-nucleotidase activity, respectively.

SDS gels of beef and rabbit myofibrils and of SR pellets were prepared by the same procedure (Porzio and Pearson, 1977). A comparison of the gels for the myofibrils and the SR (Fig. 9) showed that gels from SR contained no

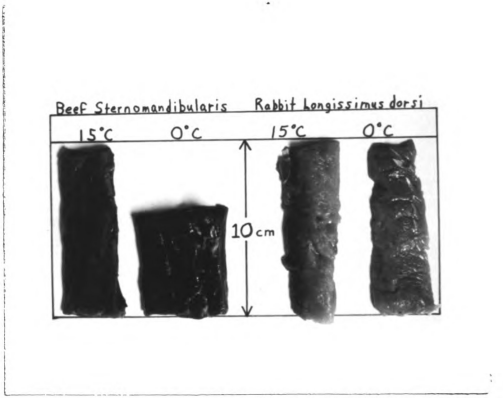

Fig. 8. Beef and rabbit muscle strips chilled to 15 or 0^{o}C immediately postmortem.

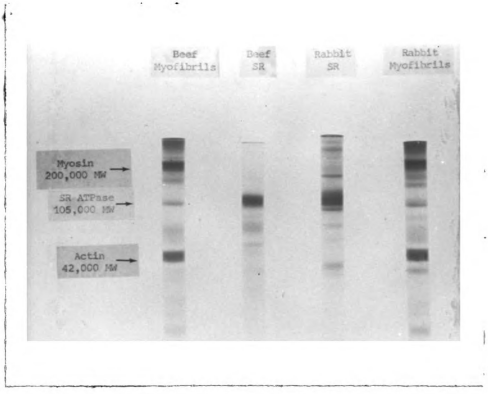

Fig. 9. SDS gels of beef and rabbit myofibrils and sarcoplasmic reticulum. The gels were 10.0% acrylamide, with 0.10% crosslinker.

myosin heavy chains (200,000 daltons) or actin (42,000 daltons), which are the predominant protein bands in SDS gels of myofibrils (Porzio and Pearson, 1977). Thus, SDS gel electrophoresis of both beef and rabbit SR preparations showed that the pellets were free of contamination from myofibrillar proteins.

In the SDS gels of the beef and rabbit SR, SR-ATPase (102,000 daltons) was the predominant band. SDS gels of beef and rabbit myofibrils also contained a band with a molecular weight of 100,000, which was identified as α-actinin (Kanda et al., 1977b). In addition to the SR-ATPase protein, the SDS gels of the SR pellets contained bands with molecular weights of 33,000, 53,000, 60,000, 93,000 and 150,000 daltons. Kanda et al. (1977b) tentatively identified the 150,000 dalton band as an M-line protein, and the 93,000 dalton component as phosphorylase a. MacLennan (1975) reported 44,000 and 55,000 dalton bands on SDS gels of rabbit SR, which he identified as calsequestrin and the high affinity Ca^{++}-binding protein, respectively. Meissner and Fleisher (1971) identified protein bands at 50,000 and 60,000 daltons in SDS gels of rabbit SR, which agrees well with the bands at 53,000 and 60,000 daltons found in the present study. Kanda et al. (1977b) also found two rabbit SR proteins with the same molecular weights. Yu et al. (1976) identified a 63,000 dalton component from rat SR as the high affinity Ca^{++} binding protein, which appears to correspond to the 60,000 dalton component in the present study. MacLennan (1975)

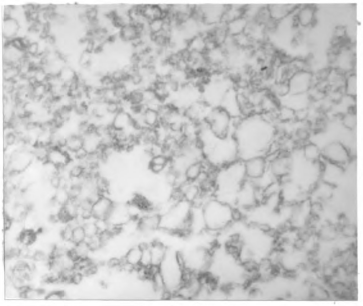

Fig. 10. Transmission electron micrograph of beef sarcoplasmic reticulum vesicles. 44,800 X.

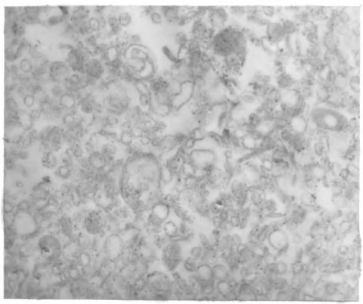

Fig. 11. Transmission electron micrograph of rabbit sarcoplasmic reticulum vesicles. 44,800 X.

identified a band at a molecular weight of 30,000 daltons in rabbit SR, which is in good agreement with the 33,000 dalton component observed in the present study. However, no function has been assigned to this protein.

Transmission electron micrographs of the beef (Fig. 10) and rabbit SR pellets (Fig. 11) showed that the pellets contained membranous vesicles with little, if any, contaminating particles. Preparations from beef and rabbit muscle were microscopically indistinguishable.

Purity and Characterization of Mitochondrial Preparations

Mitochondrial succinate oxidase activity was measured manometrically as a marker for the presence of mitochondrial membranes (King, 1967). Table 2 indicates that both beef and rabbit mitochondrial suspensions had about the same levels of succinate oxidase activity. Both suspensions oxidized about 19 µmol succinate/mg protein/minute over a one hour period. These data indicates that the beef and rabbit mitochondrial preparations contained about the same amount of mitochondrial membrane per unit protein.

Mitochondrial respiration was also measured manometrically (Ernster and Nordenbrand, 1967) to determine whether the mitochondrial suspensions contained intact mitochondria capable of respiration. Table 3 shows that the beef mitochondrial suspensions were respiring more actively than that of rabbit, indicating that the beef preparations contained more intact, respiring mitochondria per unit protein than

Table 2. Manometric Assay for Mitochondrial Succinate Oxidase
(μmol succinate oxidized/mg protein/minute)

Time (min)	Beef mitochondria	Rabbit mitochondria
10	15.06	20.02
20	20.15	23.03
30	19.91	21.49
40	19.45	19.75
50	18.83	18.27
60	18.08	17.17

Table 3. Manometric Assay for Mitochondrial Respiration (μmol O_2 uptake/mg protein/minute)

Time (min)	Beef mitochondria	Rabbit mitochondria
5	12.89	9.14
10	18.30	6.90
15	14.86	7.05
20	13.83	7.97
25	13.50	8.45
30	12.83	8.76
35	11.38	9.06
40	11.00	9.19

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that from rabbit muscle. Beef mitochondrial suspensions consumed about 13 µmol oxygen/mg protein/minute over a 40 minute period. This may be compared to about 8 µmol oxygen consumed/mg protein/minute over the same period for rabbit mitochondrial suspensions.

When viewed in the transmission electron microscope, beef and rabbit mitochondrial suspensions were strikingly different. Figure 12 shows that beef mitochondria were in the condensed conformation characteristic of state 3, i.e., actively respiring mitochondria (Lehninger, 1975). The inner mitochondrial membrane appeared dense, tightly folded and contorted. Figure 13 demonstrates that rabbit mitochondria were in the orthodox conformation, which is characteristic of state 4 or resting respiration. In this state, the inner membrane compartment completely fills the space bounded by the outer membrane (Lehninger, 1975). The micrographs show that the mitochondrial pellets consist predominantly of mitochondria, but some contaminating particles are present.

Greaser et al. (1969) also observed that mitochondrial pellets isolated by differential centrifugation contain contaminating particles. In spite of contamination, Ernster and Nordenbrand (1967) demonstrated that mitochondrial preparations isolated by differential centrifugation contained tightly coupled mitochondria suitable for use in the measurement of respiratory parameters. They further pointed out that mitochondria prepared with sucrose or other nonelectrolytes in the homogenization medium are of inferior quality,

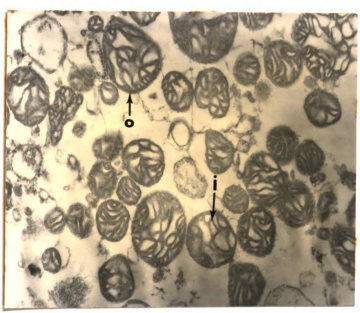

Fig. 12. Transmission electron micrograph of beef mitochondria. 28,000 X.

i = inner mitochondrial membranes

o = outer mitochondrial membranes

Fig.
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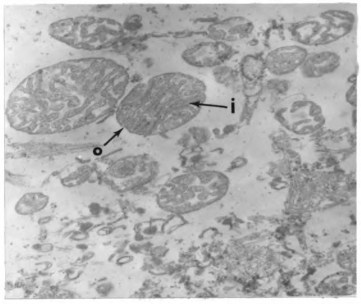

Fig. 13. Transmission electron micrograph of rabbit mitochondria. 28,000 X.

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o = outer mitochondrial membranes

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i.e., they have poor phosphorylating efficiency, high ATPase activity, and the yield of mitochondria is low. A number of workers (Greaser et al., 1969; Jacobus et al., 1975; Carafoli, 1976; Kimura and Rasmussen, 1977) have isolated mitochondria by differential centrifugation for subsequent measurements of Ca^{++} accumulation. In spite of contamination (Greaser et al., 1969; Jacobus et al., 1975), differential centrifugation has been widely used because of the speed of the isolation procedure, the high mitochondrial yield, and the good quality of the isolated mitochondria.

Yield of Sarcoplasmic Reticulum from Beef and Rabbit Muscle

In the present study, 62.2 ± 8 and 180.3 ± 40 µg of SR protein were isolated per gram of beef and rabbit muscle, respectively, using the procedure of Meissner and Fleisher (1971). Using the same procedure, Kanda (1975) obtained 42.3 ± 8 and 380 ± 40 µg SR protein/g tissue from the same muscles, respectively. Assuming that the method used for isolation of SR was quantitative, the present study indicates that the rabbit Longissimus dorsi muscle contains about 3 fold greater amounts of SR membrane than beef Sternomandibularis muscle. This is in agreement with the results of Harigaya et al. (1968) and Schiaffino et al. (1970), both of whom concluded that the SR membrane system is less well developed in red muscle fibers. However, Kanda (1975) pointed out that the beef Sternomandibularis muscle contained large amounts of connective tissue, which may reduce the

yield of SR protein from this muscle.

Martonosi (1972) reported that about 0.7 to 1.4 mg SR protein/g tissue could be isolated from red muscles, as compared to 2.5 to 4.0 mg SR protein/g of white muscle tissue. Martonosi (1972) further estimated that white muscles contain at least 5 mg SR protein/gram tissue.

Yield of Mitochondria from Beef and Rabbit Muscle

In this study, 0.637 ± 0.04 mg of mitochondrial protein were obtained per gram of beef Sternomandibularis muscle, which compares with 0.590 ± 0.07 mg of mitochondrial protein per gram of rabbit Longissimus dorsi muscle. Ernster and Nordenbrand (1967) obtained 2 to 3 mg mitochondrial protein/g rat skeletal muscle. Brand and Lehninger (1975) reported a yield of 4 mg mitochondrial protein/g rat heart, and 15 to 20 mg mitochondrial protein/g of rat liver. The low mitochondrial yields obtained in this study may be due to the homogenization method used. In view of the high connective tissue content of beef muscle, the minced muscle used in this study was homogenized with a Polytron homogenizer, instead of the all glass Potter-Elvehjem homogenizer, which was used by Ernster and Nordenbrand (1967). Polytron homogenization appeared to disrupt the tissue so that the yield of intact mitochondria sedimenting at 10,000 g was reduced.

Carafoli (1975) estimated that heart muscle contained 80 mg mitochondrial protein/g of heart tissue. Ernster and Nordenbrand (1967) obtained yields of 2 to 3 mg mitochondrial

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protein/g of rat skeletal muscle. On this basis, it may be concluded that rat skeletal muscle contains at least 6 to 8 mg mitochondrial protein/g of tissue. Martonosi (1972) estimated that white muscles contain about 5 mg SR protein/g tissue. Based on these estimates and the yield of SR and mitochondrial protein obtained in this study, it may be concluded that both red and white muscles contain at least as much mitochondrial protein as SR protein.

Gauthier (1970) pointed out that red muscles contain more mitochondria than white muscles. Muscle mitochondria may be histochemically located, using the NADH-tetrazolium reductase procedure, which specifically stains mitochondria (Engel and Brooke, 1965; Gauthier, 1970). Figures 14 and 15 show cross-sections of beef and rabbit muscle, respectively, stained by the NADH-tetrazolium reductase procedure. Red fibers, containing high mitochondrial concentrations, appear dark, while the white fibers are relatively unstained, indicating a low content of mitochondria (Engel and Brooke, 1965). A comparison of Figures 14 and 15 shows that rabbit tissue contains fewer red fibers and has a lower mitochondrial concentration than beef muscle. Although the beef Sternomandibularis is a very red muscle when viewed with the unaided eye, Figure 14 shows that it should be more properly classified as a mixed muscle, containing red, white, and intermediate fiber types. Figure 15 shows that rabbit Longissimus dorsi contains some red fibers, but the great

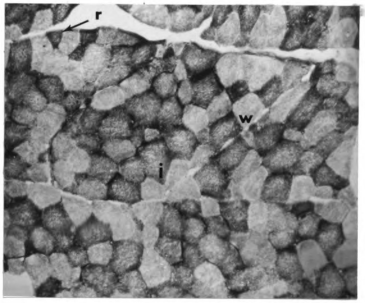

Fig. 14. Light micrograph of beef Sternomandibularis muscle 440 X.

r = red fiber

i = intermediate fiber

w = white fiber

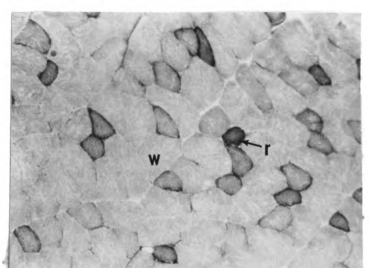

Fig. 15. Light micrograph of rabbit Longissimus dorsi muscle. 440 X.

r = red fibers

w = white fibers

majority of the muscle is composed of white fibers.

Accumualtion of Ca^{++} by Sarcoplasmic Reticulum Vesicles

Figure 16 shows that Ca^{++} accumulation by beef and rabbit SR vesicles was approximately proportional to protein concentration in a range from 0.03 to 0.3 mg of SR protein per ml of reaction mixture. Consequently subsequent experiments were conducted in this range, usually using 0.08 to 0.20 mg of SR protein per ml of reaction media.

Tables 4 and 5 show that both beef and rabbit SR vesicles accumulated more than 500 nmol Ca^{++}/mg protein/18 minutes, at pH 7.3 and 37°C. This corresponds to maximum values of 250 and 40 nmol Ca^{++}/mg protein/3 minutes reported by Sreter (1969) for SR vesicles from white and red rabbit muscle, respectively. Harigaya and Schwartz (1969) reported that SR vesicles from red rabbit muscle accumulated only 58 nmol Ca^{++}/mg protein, which agrees closely with the value of Sreter (1969). Sreter (1969) and Harigaya and Schwartz (1969) both concluded that SR vesicles from red muscles have a lower capacity for Ca^{++} accumulation than similar preparations from white muscles. The relatively high values for Ca^{++} accumulation reported in the current investivation for beef SR vesicles may be due to the fact that beef Sternomandibularis contains a significant number of intermediate and white fibers (Fig. 14). It is well known that white and intermediate fibers contain a well developed SR system (Peachey, 1970). Thus, SR vesicles

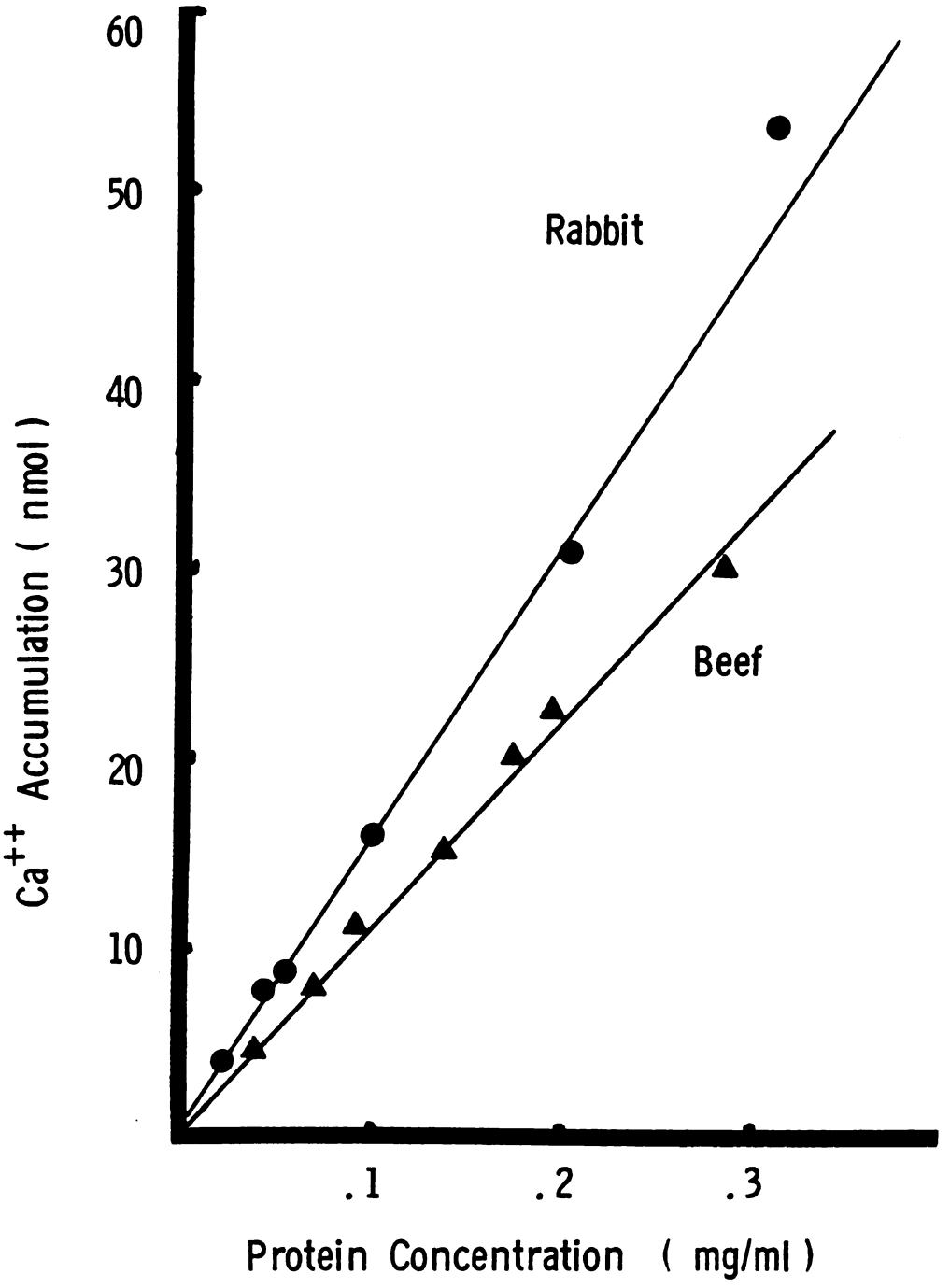

Fig. 16. Ca^{++} accumulation by sarcoplasmic reticulum vesicles at pH 7.3 and 37°C as a function of protein concentration. The reaction was allowed to proceed for 4 minutes.

derived from these fibers would be expected to have a high capacity for Ca^{++} accumulation (Sreter, 1969), which is in agreement with results from the present study.

Alternatively, the high capacities for Ca^{++} accumulation observed in the present study may be the result of a long incubation period in combination with an increasing concentration of inorganic phosphate (P_i) in the media, resulting from the release of P_i by the SR-ATPase. It is well known that Ca^{++}-precipitating agents such as P_i and oxalate greatly increase the capacity of SR vesicles to accumulate Ca^{++} (Sreter, 1969; Martonosi, 1972). Sreter (1969) observed that in the presence of oxalate, rabbit SR vesicles could accumulate more than 6,000 nmol Ca^{++}/mg protein/3 minutes.

Effects of Temperature of Ca^{++} Accumulation by Sarcoplasmic Reticulum Vesicles

Tables 4 and 5 show that beef and rabbit SR vesicles accumulated less than 70 nmol Ca^{++}/mg protein/18 minutes, when incubated at $0^{o}C$ and at pH 7.3. At $15^{o}C$, beef and rabbit SR vesicles accumulated 89 and 113 nmol Ca^{++}/mg protein/18 minutes, respectively, while at $37^{o}C$ both preparations accumulated more than 500 nmol Ca^{++}/mg protein/18 minutes. Figures 17 and 18 show that chilling the SR preparations does not inhibit subsequent Ca^{++} accumulation by the vesicles.

Table 4. Ca^{++} Accumulation and Release by Beef Sarcoplasmic Reticulum Vesicles (nmol Ca^{++}/mg protein)

Temp (°C.)	Time (minutes)	pH 7.3	6.8	6.2	5.5	5.0
37	1	77.5	70.8	73.7	--	0
37	2	90.8	119.8	54.6	69.6	8.5
37	4	190.2	198.2	74.3	--	14.3
37	8	360.1	306.0	84.9	81.7	23.8
37	13	505.8	401.6	94.6	44.0	10.3
37	18	542.5	439.0	74.8	57.0	0
37 + 0[1]	8 + 5	298.7	280.7	23.3	9.4	32.8
37 + 0[2]	8 + 10	294.7	238.7	35.4	47.6	4.9
37[3]	8 at pH 7.3 +10 at pH 5.0					0
15	1	--	50.1	54.1	--	--
15	2	72.2	47.5	53.3	57.1	4.9
15	4	78.3	16.7	63.4	48.7	--
15	8	85.8	40.5	77.6	22.9	0
15	13	80.4	54.6	67.6	115.0	24.7
15	18	89.4	34.7	97.6	51.2	0
15 + 0	8 + 5	60.4	22.2	75.4	7.6	0
15 + 0	8 + 10	54.0	25.8	51.7	21.1	16.7
0	1	21.4	0	--	--	--
0	2	27.6	4.5	36.7	0	0
0	4	42.7	0	--	--	--
0	8	42.2	0	23.1	0	0
0	13	49.3	0	37.7	9.8	0
0	18	61.1	7.6	0	34.6	0
0 + 37	8 + 5	267.4	180.0	118.5	34.6	0
0 + 37	8 + 10	384.1	279.2	138.0	49.0	0

[1] Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 5 minutes at 0°C

[2] Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 10 minutes at 0°C

[3] Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 10 minutes at pH 5.0 and 37°C

Table 5. Ca^{++} Accumulation and Release by Rabbit Sarcoplasmic Reticulum Vesicles
(nmol Ca^{++}/mg protein)

Temp. (°C.)	Time (minutes)	pH 7.3	6.8	6.2	5.5	5.0
37	1	70.8	115.4	153.4	55.7	30.9
37	2	111.8	138.8	128.8	55.8	22.1
37	4	210.7	202.5	102.7	28.1	20.3
37	8	348.9	320.8	74.4	16.7	13.6
37	13	379.0	282.1	36.3	15.8	18.2
37	18	543.5	209.2	37.2	1.8	24.0
37 + 0[1]	8 + 5	271.0	190.5	39.1	0.0	24.2
37 + 0[2]	8 + 10	242.1	230.0	33.3	1.9	31.9
37[3]	8 at pH 7.3 +10 at pH 5.0[3]					18.7
15	1	72.7	76.3	115.3	25.0	14.2
15	2	71.4	87.4	116.9	25.1	14.9
15	4	79.8	93.9	129.3	36.1	30.1
15	8	92.3	100.5	130.6	38.0	52.1
15	13	99.2	95.9	130.6	43.0	51.7
15	18	113.1	121.7	125.0	42.3	54.2
15 + 0	8 + 5	78.7	87.9	91.9	30.0	--
15 + 0	8 + 10	81.8	80.3	86.2	19.2	48.9
0	1	45.2	53.3	42.0	5.9	0
0	2	50.0	51.8	57.7	8.7	0
0	4	64.4	56.3	66.4	12.2	0
0	8	65.7	64.7	73.1	11.2	0
0	13	66.8	57.3	78.1	23.4	0
0	18	58.0	41.5	89.0	20.0	0
0 + 37	8 + 5	162.4	273.8	96.6	26.1	0
0 + 37	8 + 10	297.0	310.7	66.0	20.9	0

[1]Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 5 minutes at 0°C

[2]Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 10 minutes at 0°C

[3]Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 10 minutes at pH 5.0 and 37°C

When beef or rabbit SR vesicles were warmed to 37°C after being chilled at 0°C for 8 minutes, both preparations accumulated more than 300 nmol Ca^{++}/mg protein in 10 minutes. These results show that Ca^{++} accumulation by SR vesicles is strongly temperature dependent. Several previous investigations have reported similar results (Martonosi and Feretos, 1964; Sreter, 1969; La Court, 1971; Kanda, 1975). In contrast, Sreter (1969) found that the Ca^{++} accumulating ability of rabbit SR vesicles increases with temperature over the range of 0 to 25°C, but at 35°C, the initial uptake was followed by release of Ca^{++}. However, no explanation for the release of Ca^{++} at 35°C was provided (Sreter, 1969).

Effects of Temperature on Ca^{++} Release by Sarcoplasmic Reticulum Vesicles

Figures 17 and 18 show that after SR vesicles have accumulated Ca^{++} for 8 minutes at pH 7.3 and 37°C, chilling the media to 0°C completely inhibits further accumulation. In fact, the drop in temperature from 37 to 0°C caused release of some of the accumulated Ca^{++}. After 10 minutes at 0°C, beef and rabbit SR vesicles released 66 and 106 nmol Ca^{++}/mg protein (Tables 4 and 5, respectively). This corresponds to 18 and 30 percent of the total accumulated Ca^{++}. Chilling beef and rabbit SR vesicles from 15 to 0°C caused the release of 31 and 11 nmol Ca^{++}/mg protein/10 minutes, corresponding to a release of 36 and 12% of the initial Ca^{++} load, respectively.

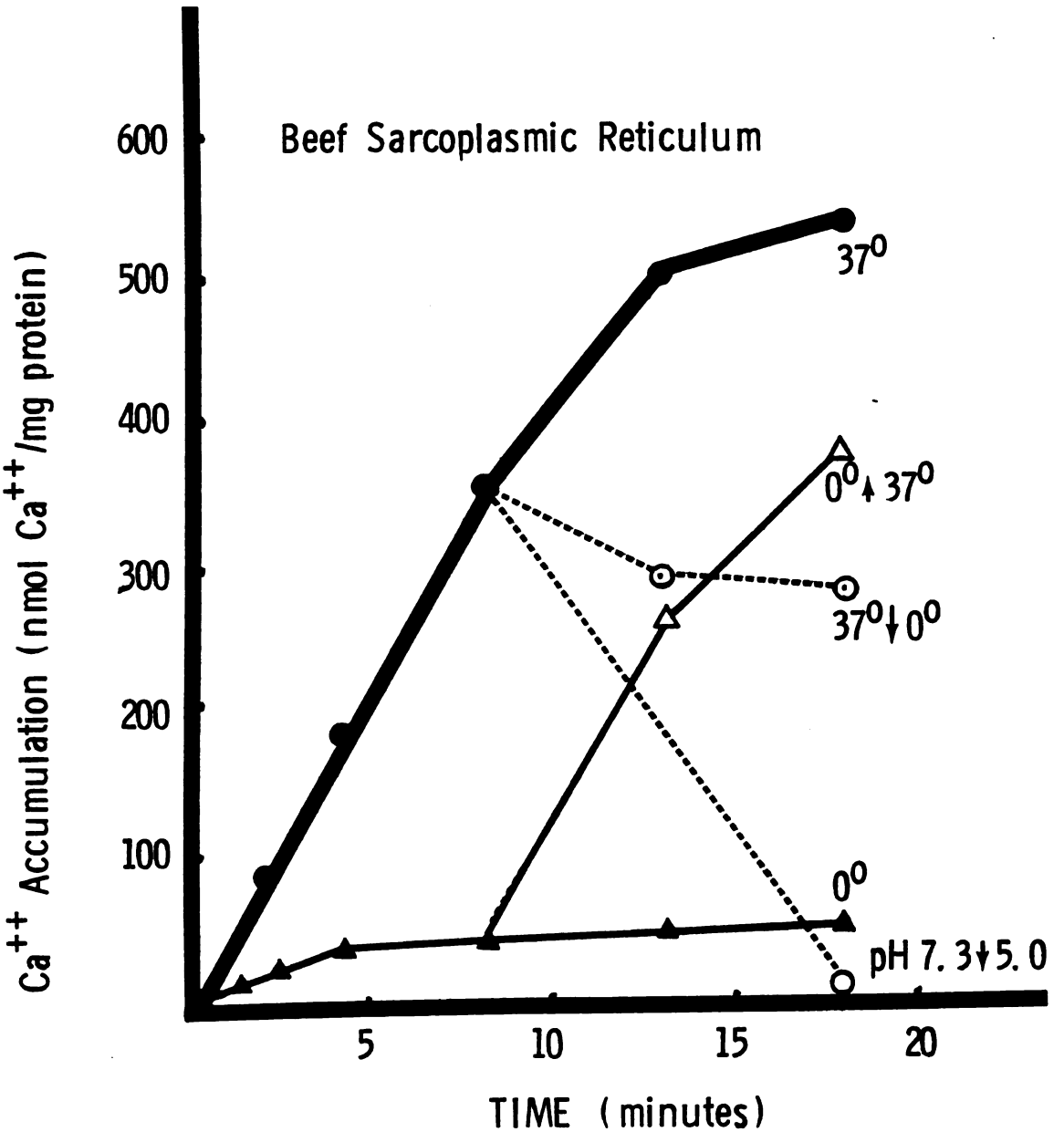

Fig. 17. Ca^{++} accumulation and release by beef sarcoplasmic reticulum vesicles as affected by temperature and pH.

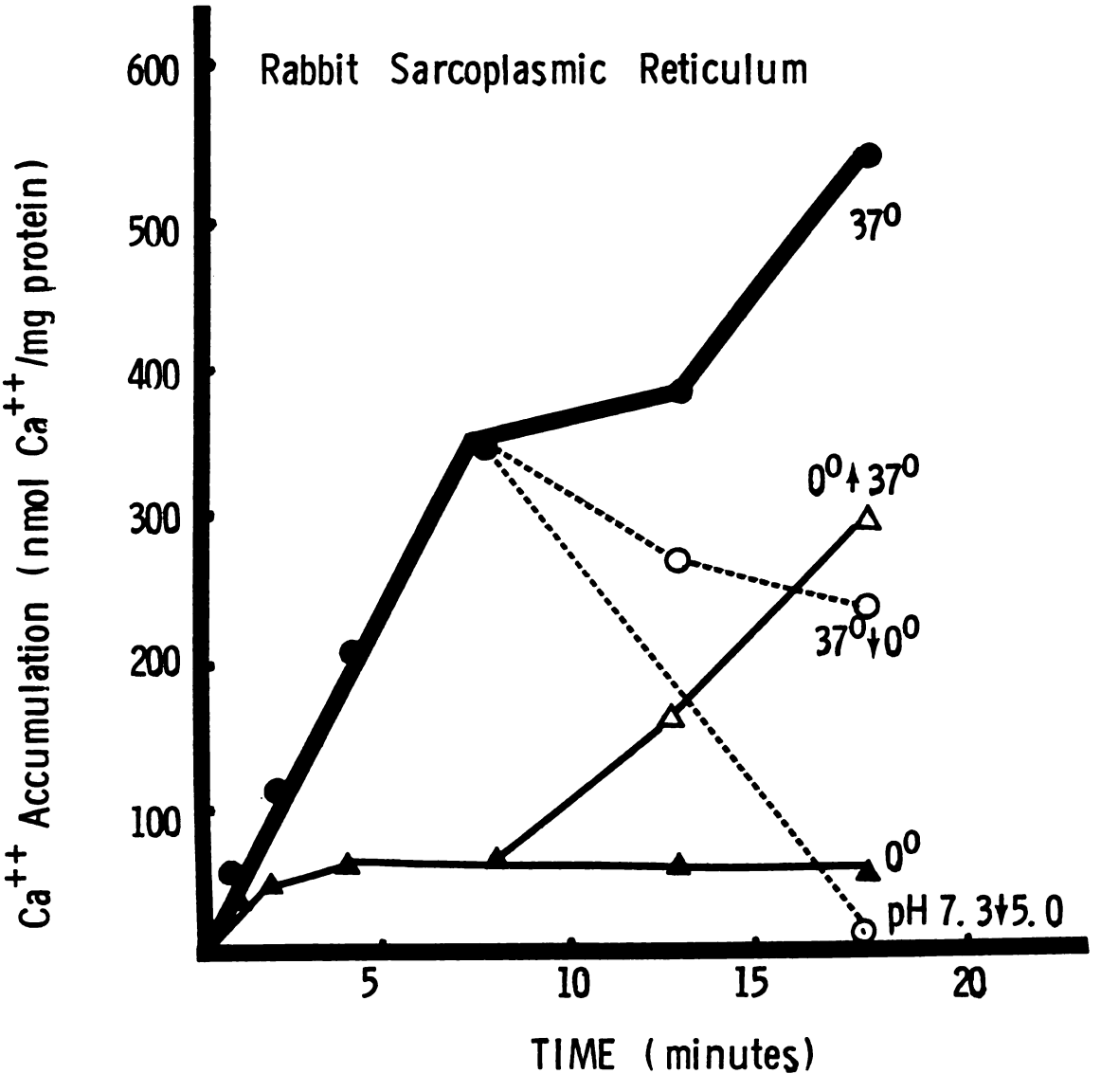

Fig. 18. Ca^{++} accumulation and release by rabbit sarcoplasmic reticulum vesicles as affected by temperature and pH.

Kanda (1975) also observed the release of Ca^{++} from preloaded chilled SR vesicles. These findings support the conclusions of Pearson et al. (1973) and Davey and Gilbert (1974), both of whom concluded that chilling the SR causes a release of Ca^{++}, which in turn results in muscle cold shortening. It must be pointed out, however, that the fragmented SR vesicles may be more susceptible to Ca^{++} leakage than the intact SR membrane system. Consequently, chilled SR vesicles may release more Ca^{++} than would be the case for the intact SR system. However, there is little doubt that chilling SR vesicles or intact muscle to $0^{o}C$ virtually abolishes Ca^{++} accumulation. Furthermore, results of the present study suggest that Ca^{++} is released from chilled SR membranes, thus contributing to cold shortening.

Effects of pH on Ca^{++} Accumulation by Sarcoplasmic Reticulum Vesicles

Tables 4 and 5 show that beef and rabbit SR vesicles, respectively, accumulated 300-500nmol Ca^{++}/mg protein at pH 7.3 or pH 6.8 at $37^{o}C$. At pH 6.2 and $37^{o}C$, beef and rabbit SR vesicles accumulated a maximum of 94 and 153nmol Ca^{++}/mg protein, respectively. At pH 5.5 or below and at $37^{o}C$, neither SR preparation accumulated more than 82nmol Ca^{++}/mg protein. At both 15 and $0^{o}C$, maximum Ca^{++} accumulation was generally less than 100nmol Ca^{++}/mg protein in both rabbit and beef SR vesicles, regardless of the pH. Even at these reduced temperatures, however, Ca^{++} accumulation by SR

vesicles was much less at pH 5.5 or 5.0 than at the higher pH values. The data indicate that at pH values of 6.2 or below, Ca^{++} accumulation by both beef and rabbit SR vesicles is markedly reduced. Several other investigators have also observed that Ca^{++} accumulation by SR vesicles is greatly reduced at pH 5.0 (Sreter, 1969; La Court, 1971; Kanda, 1975; Berman et al., 1977). Berman et al. (1977) further reported that brief exposure of SR vesicles to pH values in the range of 5.5 to 6.0 caused rapid and irreversible inactivation of Ca^{++} accumulation.

In contrast to the results of the present investigation and several other studies (La Court, 1971; Kanda, 1975; Berman et al., 1977), Sreter (1969) reported that optimum Ca^{++} accumulation by rabbit SR vesicles occurred in the pH range 5.6 to 6.5. Although Sreter (1969) observed an inhibition of Ca^{++} accumulation at pH 5.0, which agrees with the results of the present study, he concluded that at pH 7.0 Ca^{++} uptake was significantly decreased and at pH 7.4, Ca^{++} uptake was only 40% of maximum. This is surprising since Sreter's (1969) results indicate that Ca^{++} accumulation is minimum at physiological pH, and is maximum at pH values existing in muscle under rigor conditions.

The results of the present study clearly show that low pH values in the range 5.0 to 5.5 inhibit Ca^{++} accumulation by SR vesicles. This suggests that white muscle may be less susceptible to cold shortening due to its rapid rate of post-mortem glycolysis, sometimes resulting in pH values of 5.5

or less in only 30 minutes postmorten (Kastenschmidt, 1970).

Effects of pH on Ca^{++} Release by Sarcoplasmic Reticulum Vesicles

Figures 17 and 18 show that beef and rabbit SR vesicles preloaded with 300 to 375 nmol Ca^{++}/mg protein released almost all of the accumulated Ca^{++} when the pH of the media was lowered to 5.0. The release of Ca^{++} was rapid, occurring in a 10 minute period. Thus, the results of the present study show that at low pH values in the range of 5.0 to 5.5, Ca^{++} accumulation by the SR vesicles is reduced, and significant amounts of Ca^{++} are released.

In postmortem muscle, glycolysis lowers the muscle pH to values in the range of 5.0 to 6.0 (Kastenschmidt, 1970). Busch et al. (1967) reported that rigor shortening occurs at pH values below 6.0. Goll et al. (1971) and La Court (1971) both concluded that postmortem proteolysis is responsible for inactivation of the SR membrane, causing Ca^{++} release and consequently rigor shortening. However, results of the present study indicate that the low postmortem pH alone is sufficient to inactivate the SR membrane and inhibit Ca^{++} accumulation. The results of Berman et al. (1977) and Kanda (1975) support this conclusion.

Effects of Protein Concentration on Mitochondrial Ca^{++} Accumulation

Figure 19 shows that mitochondrial Ca^{++} accumulation was not strictly proportional to protein concentration.

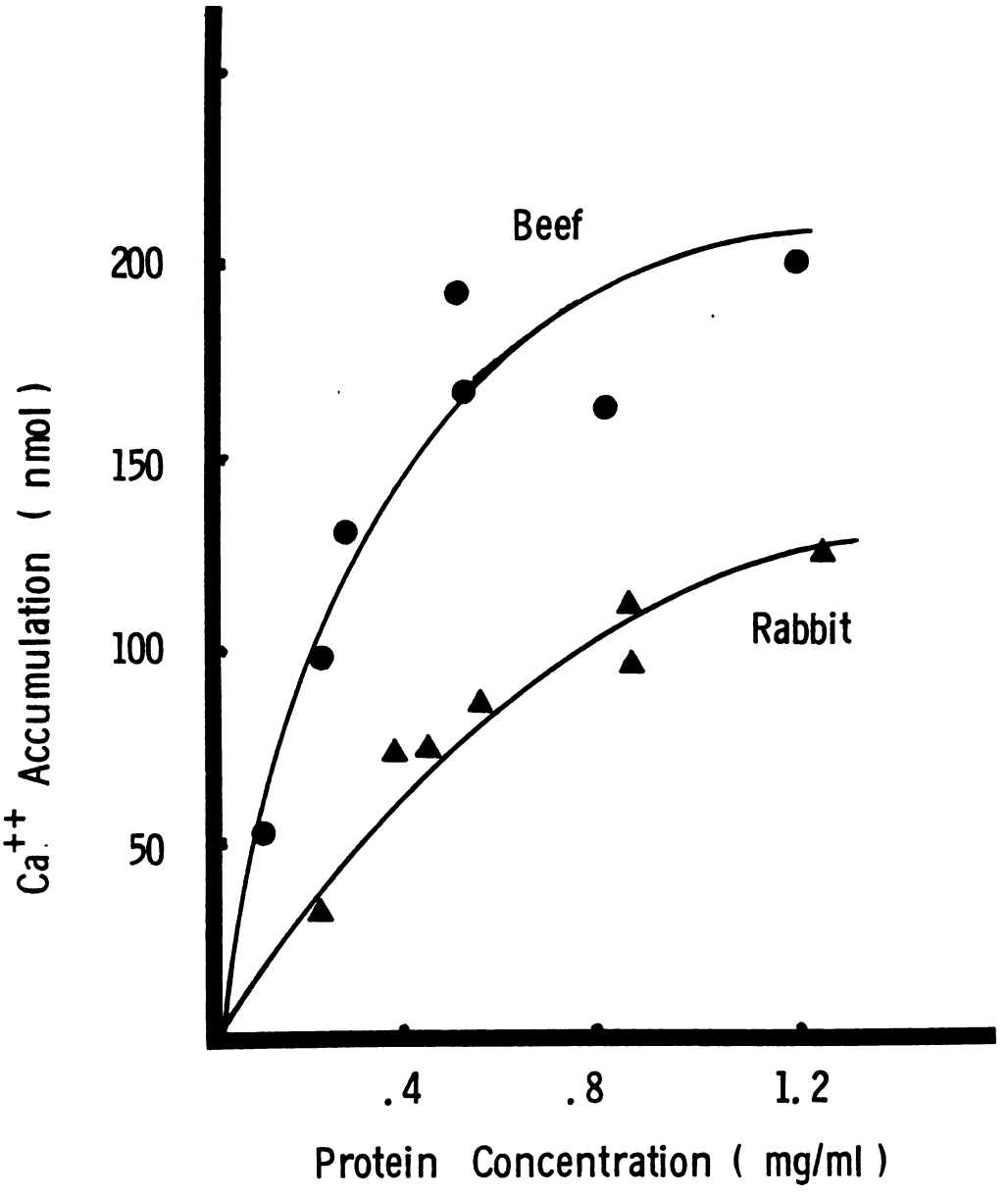

Fig. 19. Ca^{++} accumulation by mitochondria at pH 7.3 and 37°C as a function of protein concentration. The reaction was allowed to proceed for 4 minutes.

Therefore, all subsequent experiments were conducted using 0.5 to 0.8 mg mitochondrial protein/ml reaction mixture in order to eliminate the effects of protein concentration on Ca^{++} accumulation. Several investigators (Drahota et al., 1965; Malstrom and Carafoli, 1975; Jacobus et al., 1975) used higher protein concentrations, ranging from 1.45 to 4.0 mg protein/ml, in assays for mitochondrial Ca^{++} accumulation. However, it was observed in the present study that at protein concentrations greater than 1.0 to 1.4 mg protein/ml, the pores of the millipore filters were rapidly stopped. Consequently, a sufficient amount of filtrate could not be obtained for analysis. At protein concentrations of 0.5 to 0.8 mg/ml, the solutions could be rapidly filtered, and mitochondrial Ca^{++} accumulation was significant (Fig. 19).

Role of ATP in Mitochondrial Ca^{++} Accumulation

In order to accumulate more than 60 nmol Ca^{++}/mg protein, beef (Fig. 20) and rabbit mitochondria (Fig. 21) required the presence of 3.0 mM ATP in the reaction media. This is in agreement with the results of Chance (1964), who reported that rat liver mitochondria had a limited capacity for Ca^{++} accumulation in the absence of ATP or P_i. He reported that under these conditions Ca^{++} accumulation did not exceed 100 nmol/mg protein. On the other hand, Drahota et al. (1965) observed that rat liver mitochondria in the presence of ATP could accumulate more than 500 nmol Ca^{++}/mg protein, which

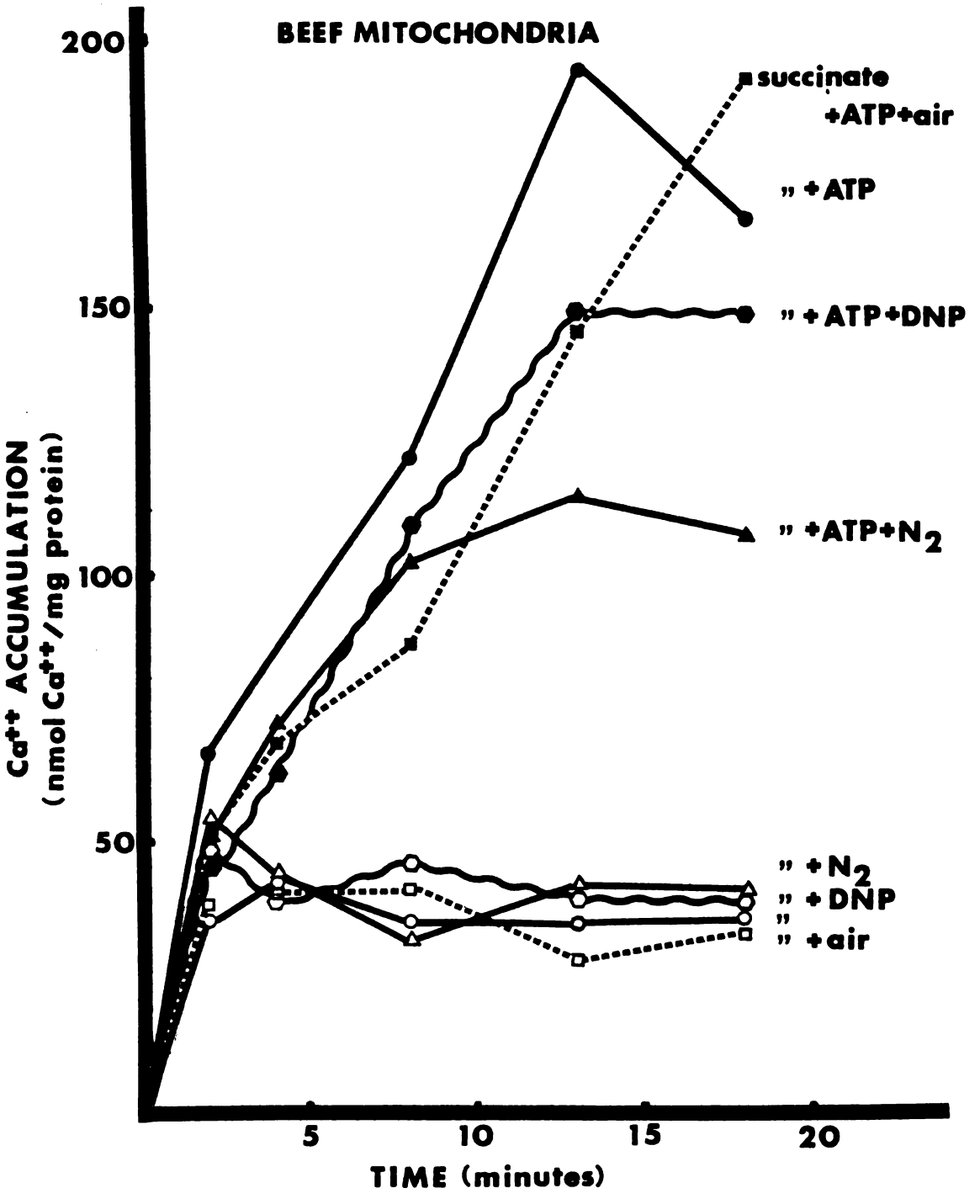

Fig. 20. Ca^{++} accumulation by beef mitochondria as affected by ATP, 2,4-dinitrophenol and anaerobic conditions. Incubation was carried out at pH 7.3 and 37°C.

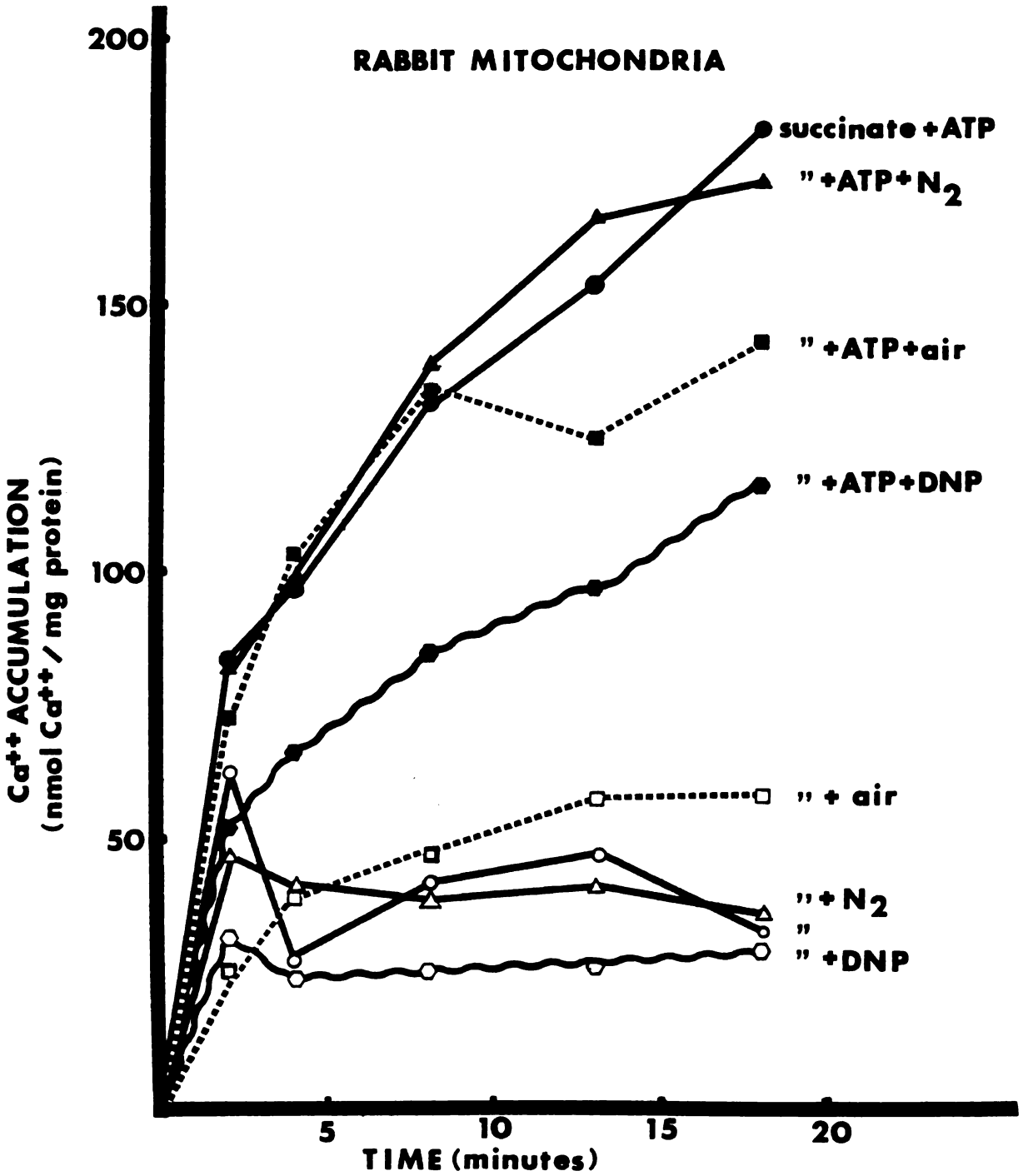

Fig.21. Ca^{++} accumulation by rabbit mitochondria as affected by ATP, 2,4-dinitrophenol and anaerobic conditions. Incubation was carried out at pH 7.3 and 37°C.

compares reasonably well with the values of over 400nmol Ca^{++}/ mg protein obtained in the present study (Table 6 and 7).

There are two possible mechanisms by which ATP may support mitochondrial Ca^{++} accumulation. First, the presence of ATP in the media may be necessary for the retention of Ca^{++} accumulated by a respiration-linked process, as was found to be the case for Ca^{++} accumulated by rat liver mitochondria (Carafoli et al., 1965; Kimura and Rasmussen, 1977). Alternatively, Ca^{++} accumulation may actually be supported by ATP hydrolysis in the absence of mitochondrial respiration, as was demonstrated in rat liver mitochondria by Bielawski and Lehninger (1966) and Brand and Lehninger (1975). Unfortunately, the mechanism by which ATP supports Ca^{++} accumulation by muscle mitochondria was not conclusively determined by the present study. However, evidence to be presented later suggests that both mechanisms could possibly be important for Ca^{++} accumulation by muscle mitochondria.

Effects of Nitrogen, Air, or 2,4-dinitrophenol on Mitochondrial Ca^{++} Accumulation

Buege and Marsh (1975) observed that chilled muscle strips cold shortened in the presence of mitochondrial uncoupling agents, such as 2,4-dinitrophenol, or in an anaerobic atmosphere. However, aerobic conditions inhibited cold shortening. They postulated that these conditions influence cold shortening by affecting mitochondrial Ca^{++} accumulation or retention.

Table 6. Ca^{++} Accumulation and Release by Beef Mitochondria (nmol Ca^{++}/mg protein)

Temp. (°C.)	Time (minutes)	pH 7.3	6.8	6.2	5.5	5.0
37	2	381.4	190.9	81.2	37.6	--
37	4	374.7	278.5	164.7	32.0	28.2
37	8	337.2	294.0	196.1	26.1	26.2
37	13	390.2	302.1	256.1	37.6	21.8
37	18	259.2	305.5	280.1	70.2	19.1
37 + 0[1]	8 + 5	290.2	289.5	327.2	--	--
37 + 0[2]	8 + 10	294.4	270.6	326.7	77.4	32.3
37[3]	8 at pH 7.3					71.6
37[4]	8 + 10 N_2 gas	242.2				
15	2	--	--	--	--	--
15	4	179.7	186.2	80.7	54.4	0
15	8	249.4	190.3	99.8	21.1	0
15	13	260.7	233.4	118.1	33.9	8.7
15	18	311.2	244.9	159.5	19.9	0
15	8 + 5 at 0°	--	183.5	104.1	--	--
15	8 + 10 at 0°	244.8	213.0	119.2	--	0
0	2	44.1	36.1	12.3	0	0
0	4	162.1	38.7	14.4	5.8	0
0	8	134.0	50.2	15.5	8.3	0
0	13	65.4	38.5	35.6	0	0
0	18	59.0	59.2	55.4	0	0
0	8 + 5 at 37°	--	391.5	295.8	--	0
0	8 + 10 at 37°	298.5	390.7	286.3	36.5	0

[1] Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 5 minutes at 0°c

[2] Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 10 minutes at 0°C

[3] Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 10 minutes at pH 5.0 and 37°C

[4] Incubation at pH 7.3 and 37°C for 8 minutes, followed by bubbling nitrogen gas through the medium for 10 minutes at pH 7.3 and 37°C

Table 7. Ca^{++} Accumulation and Release by Rabbit Mitochondria
(nmol Ca^{++}/mg protein)

Temp. (°C.)	Time (minutes)	pH 7.3	6.8	6.2	5.5	5.0
37	2	147.4	75.6	78.5	95.4	--
37	4	202.3	98.7	115.7	78.5	--
37	8	282.6	169.5	164.6	65.1	32.7
37	13	371.0	180.7	139.7	62.7	30.7
37	18	454.7	231.1	137.2	73.7	15.9
37 + 0[1]	8 + 5	--	164.0	153.9	--	--
37 + 0[2]	8 + 10	234.4	218.4	196.3	93.6	10.2
37[3]	8 + 10 at pH 5.0					69.7
37[4]	8 + 10 N_2 gas	264.0				
15	2	90.0	105.9	--	--	0
15	4	111.0	70.7	106.7	32.1	0
15	8	164.8	67.4	96.5	38.4	0
15	13	175.3	70.7	96.7	44.5	0
15	18	164.2	83.9	134.2	51.0	0
15	8 + 5	177.7	70.8	--	42.1	0
15	8 + 10	164.6	76.8	118.1	35.6	0
0	2	78.8	--	--	--	0
0	4	88.2	51.7	45.1	10.1	0
0	8	80.5	43.3	56.8	11.7	0
0	13	76.7	65.7	--	--	0
0	18	81.3	73.7	76.4	7.0	0
0	8 + 5 at 37°	266.7	168.0	--	--	--
0	8 + 10 at 37°	359.2	177.2	176.0	53.2	--

[1]Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 5 minutes at 0°C

[2]Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 10 minutes at 0°C

[3]Incubation at pH 7.3 and 37°C for 8 minutes, followed by incubation for 10 minutes at pH 5.0 and 37°C

[4]Incubation at pH 7.3 and 37°C for 8 minutes, followed by bubbling nitrogen gas through the medium for 10 minutes at pH 7.3 and 37°C

In the present study, direct measurements of Ca^{++} accumulation and release by muscle mitochondria were conducted, using conditions similar to those described by Buege and Marsh (1975). Figure 20 shows that Ca^{++} accumulation by beef mitochondria was somewhat reduced in the presence of nitrogen at incubation times of either 13 or 18 minutes. Under the same conditions, on the other hand, Ca^{++} accumulation by rabbit mitochondria (Fig. 21) was not significantly affected by the presence of nitrogen. Bubbling air through the mitochondrial preparations did not increase mitochondrial Ca^{++} accumulation as compared to preparations incubated in the absence of bubbling air or nitrogen gas (Fig. 20 and 21).

The mitochondrial uncoupler, 2,4-dinitrophenol, partially inhibited Ca^{++} accumulation by rabbit mitochondria (Fig. 21), but did not significantly inhibit Ca^{++} accumulation by beef mitochondria (Fig. 20). However, Figures 20 and 21 show that both beef and rabbit mitochondria accumulated significant amounts of Ca^{++}, with values in excess of 100 nmol Ca^{++}/mg protein/18 minutes, when incubated in the presence of a mitochondrial uncoupler (2,4-dinitrophenol) or an anaerobic environment (bubbling nitrogen gas).

In contrast to the results of the present experiment, Carafoli (1976) stated that 2,4-dinitrophenol completely inhibited Ca^{++} accumulation and initiated rapid release of Ca^{++} from rat liver mitochondria. Sordahl (1974) also observed an inhibition of Ca^{++} accumulation by rabbit heart

mitochondria if the medium was allowed to become anaerobic. Thus, results of the present investigation show that muscle mitochondria do not respond to mitochondrial uncouplers or anaerobic conditions in the same manner as mitochondria from rat liver or rabbit heart. Furthermore, results obtained in the present study indicate that the response of muscle mitochondria to anaerobic conditions is not as pronounced as might be expected based on the hypothesis of Buege and Marsh (1975).

The results of the present study suggest that at least a portion of the mitochondrial Ca^{++} uptake for both beef and rabbit mitochondria is accumulated in a respiration-independent process, perhaps supported by ATP hydrolysis. Ca^{++} accumulation supported by ATP hydrolysis would not be dependent upon mitochondrial respiration, and thus, anaerobic conditions would not lead to Ca^{++} release. This is supported by the results of Weber *et al.* (1966). They observed that mitochondrial inhibitors decreased Ca^{++} accumulation by only 10% for rabbit muscle mitochondria, while the same inhibitors completely inhibited mitochondrial Ca^{++} accumulation in tissues other than muscle. Greaser *et al.* (1969) similarly observed that 5 mM sodium azide, which blocks mitochondrial Ca^{++} accumulation in most tissues (Fanburg *et al.*, 1965), inhibited only 5% of the Ca^{++} accumulating ability of pig muscle mitochondria.

Greaser *et al.* (1969) further suggested that contaminating SR fragments may play an important role in Ca^{++}

accumulation by muscle mitochondrial fractions. However, since muscle mitochondrial preparations (Tables 6 and 7) accumulate Ca^{++} at about the same level as pure SR preparations (Table 4 and 5), it is improbable that SR contamination could account for the high levels of Ca^{++} accumulated by mitochondria in this study. Thus, the evidence points towards muscle mitochondrial accumulation of Ca^{++} supported by ATP hydrolysis.

Effects of Nitrogen on Mitochondrial Ca^{++} Release

Both beef and rabbit mitochondrial suspensions released a portion of their previously accumulated Ca^{++} when nitrogen gas was bubbled through the medium. Figure 22 shows that beef mitochondrial suspensions released about 95 nmol Ca^{++}/mg protein/10 minutes as compared to only 18 nmol Ca^{++} for rabbit mitochondrial suspensions (Fig. 23). Expressed on a percentage basis, the beef mitochondrial suspensions released 28% of their accumulated Ca^{++} as compared to a loss of only 6% from rabbit mitochondrial suspensions. The greater proportion of Ca^{++} released by beef muscle mitochondria may be due to the higher levels of Ca^{++} accumulated prior to imposing anaerobic conditions. Initially, beef mitochondria contained 337 nmol Ca^{++}/mg protein as compared to 282 for rabbit mitochondria. The greater loss of Ca^{++} by beef mitochondria is supported by the results of Jacobus *et al.* (1975), who concluded that the amount of Ca^{++} released is proportional to the initial Ca^{++} load of the mitochondria. Martonosi and

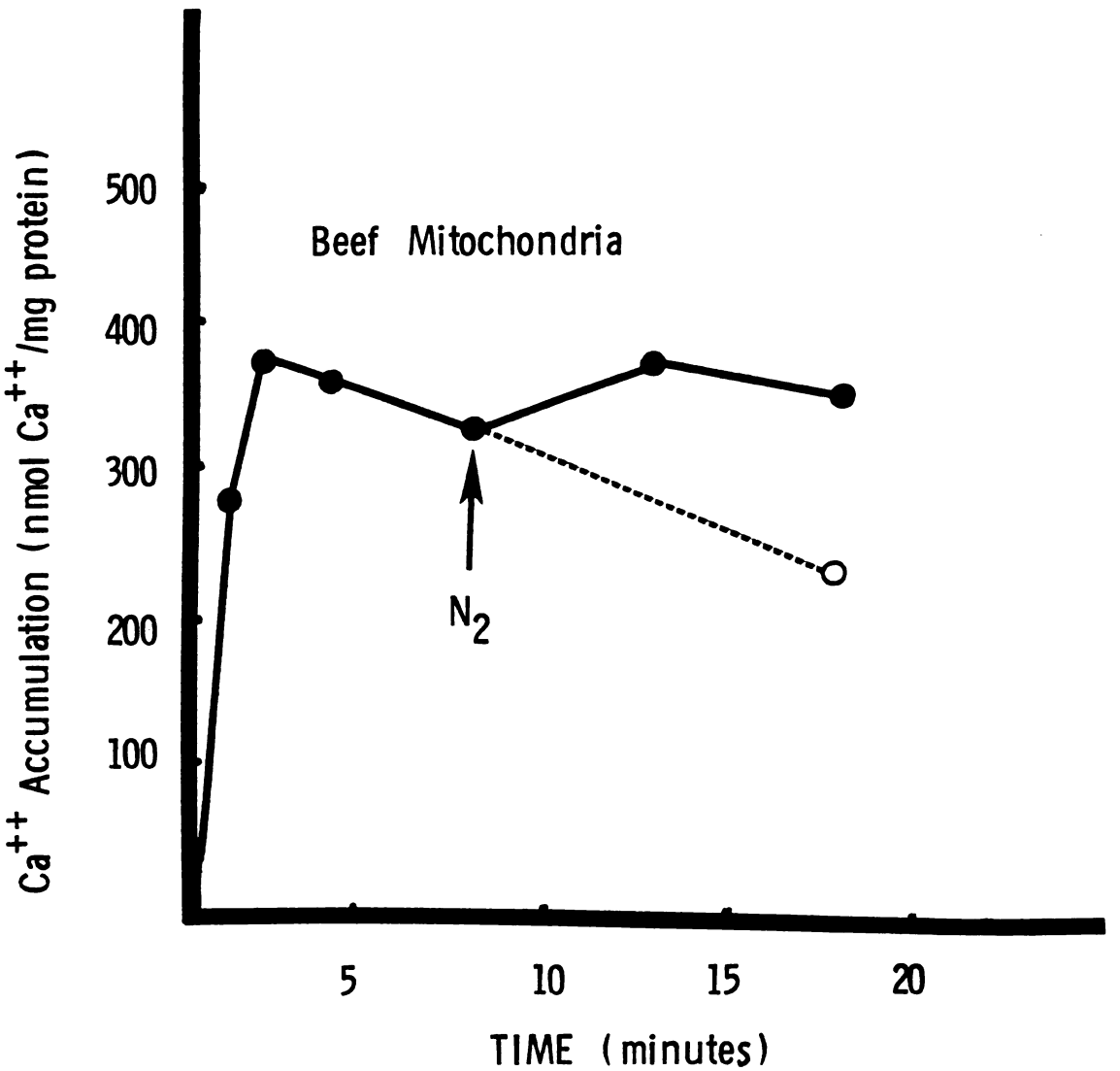

Fig. 22. Ca^{++} release by beef mitochondria at pH 7.3 and 37°C in the presence of nitrogen.

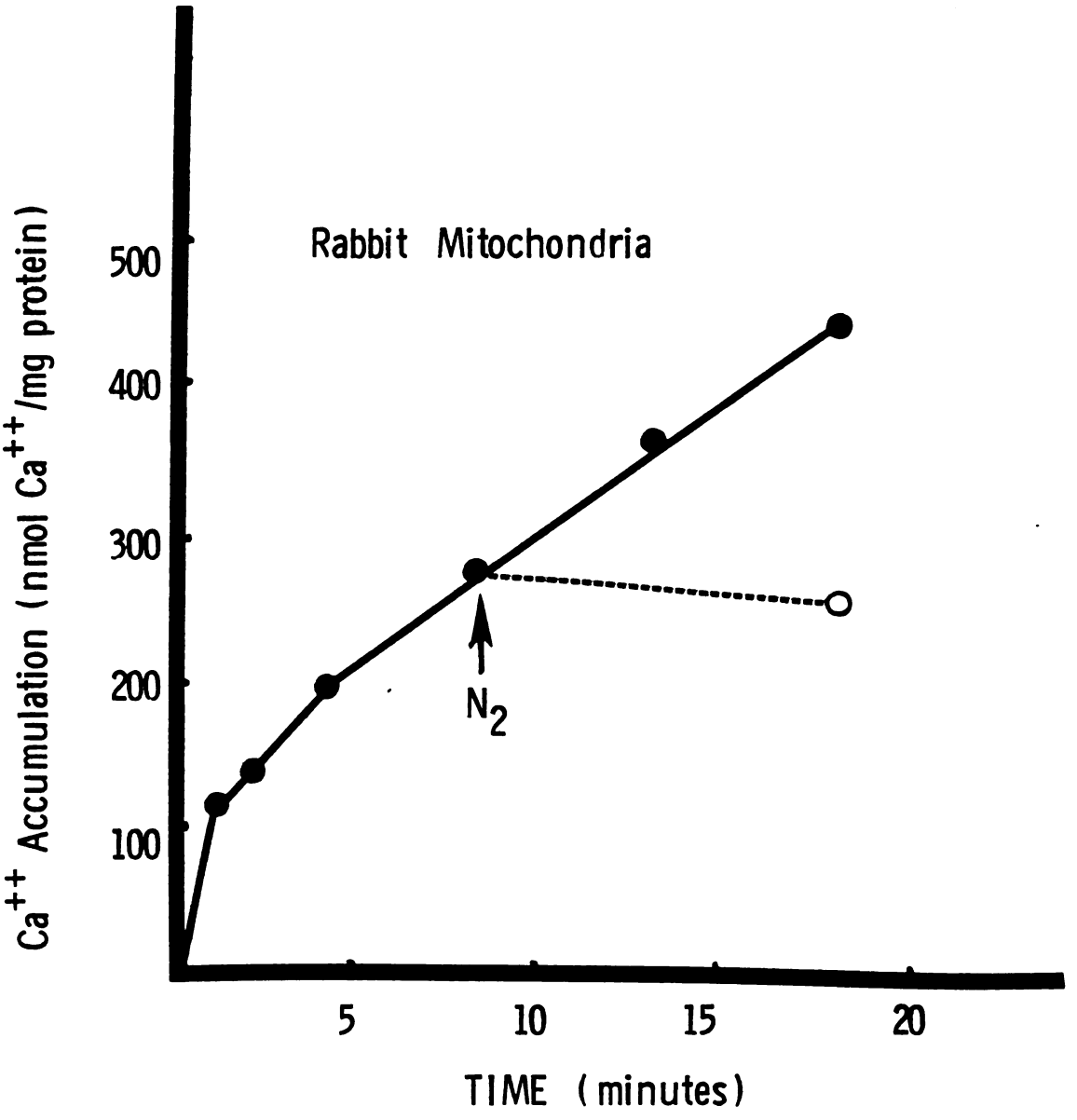

Fig. 23. Ca^{++} release by rabbit mitochondria at pH 7.3 and 37°C in the presence of nitrogen.

Feretos (1964) had previously reached the same conclusion using SR vesicles.

Alternatively, the greater proportion of Ca^{++} released by beef mitochondria may be due to the fact that more Ca^{++} is bound to beef mitochondria in a respiration linked manner. Electron micrographs showed that beef mitochondria were in an actively respiring state (Fig. 12), while rabbit mitochondria were in a state of resting respiration (Fig. 13). Manometric measurements also showed that beef mitochondrial suspensions (Table 2) were more actively respiring than rabbit mitochondria (Table 3).

Figures 20 and 21 show that the presence of nitrogen reduced Ca^{++} accumulation by beef mitochondria, while having no effect on rabbit mitochondria. These results suggest that beef mitochondria accumulate Ca^{++} in a respiration linked manner, while rabbit mitochondria accumulate Ca^{++} supported by ATP hydrolysis. Consequently, beef mitochondria would be more susceptible to anaerobic conditions, thus releasing more Ca^{++}.

Sordahl (1974) also observed that anaerobic conditions initiated the release of Ca^{++} mitochondrial preparations. He found that rabbit heart mitochondria released 70% of the accumulated Ca^{++} when the medium became anaerobic. Drahota et al. (1965) reported that rat liver mitochondria released Ca^{++} in the absence of a respiratory substrate. These studies suggest that mitochondrial respiration is necessary for retention of Ca^{++}, at least in rabbit heart and rat

liver mitochondria. Results of the present study also indicated that respiration may be necessary for retention of a part of the Ca^{++} accumulated by muscle mitochondria, especially in the case of beef muscle mitochondria.

It is unlikely that intact muscle mitochondria contain as much Ca^{++} as they are able to accumulate in an *in vitro* system. However, Carafoli (1975) pointed out that very small amounts of Ca^{++} are necessary to initiate muscle contraction. He stated that muscle contraction occurs when the Ca^{++} concentration in the myofibrillar region increases from 10^{-7} to 10^{-5} M, which corresponds to an increase from 0.1 to 10.0 nmolCa^{++}/g muscle tissue. The results of this *in vitro* study show that anaerobic conditions, low pH and low temperature can initiate the release of more than enough Ca^{++} to cause cold shortening.

Influence of Temperature on Mitochondrial Ca^{++} Accumulation

Tables 6 and 7 show that mitochondrial Ca^{++} accumulation is temperature dependent. At 37°C and pH 7.3, both beef (Table 6) and rabbit mitochondrial suspensions (Table 7) accumulated more than 390 nmolCa^{++}/mg protein/18 minutes. At 15°C, beef and rabbit mitochondrial suspensions accumulated 311 and 163 nmolCa^{++}/mg protein/18 minutes, while at 0°C the suspensions accumulated only 69 and 81 nmolCa^{++}/mg protein, respectively. Thus, muscle mitochondrial Ca^{++} accumulation is significantly reduced at low temperatures such as occur in conditions favoring cold shortening.

Reed and Bygrave (1975) also observed that mitochondrial Ca^{++} accumulation was temperature dependent, and suggested that kinetic studies of mitochondrial Ca^{++} accumulation be conducted at reduced temperatures in order to obtain more accurate estimates of initial rate parameters. The results of the present investigation support the theory that low temperatures reduce Ca^{++} accumulation by mitochondria.

Influence of Temperature on Mitochondrial Ca^{++} Release

Figures 24 and 25 demonstrate that preloaded muscle mitochondrial preparations at 37°C and pH 7.3 released small amounts of Ca^{++} when the suspension was chilled to 0°C. Beef (Fig. 24) and rabbit mitochondrial suspensions (Fig. 25) released 43 and 48nmol Ca^{++}/mg protein/10 minutes, which corresponds to the release of 13 and 17% of the initial Ca^{++} load, respectively. However, the effects of chilling on the mitochondrial preparations were reversible by warming the suspensions to 37°C. Figures 24 and 25 also show that both beef and rabbit mitochondrial suspensions were able to accumulate more than 300nmol Ca^{++}/mg protein/10 minutes, when warmed to 37°C after previous chilling for 8 minutes at 0°C. This clearly demonstrates the reversible nature of Ca^{++} accumulation with temperature.

Although the present study shows that chilled mitochondria release Ca^{++}, Drahota *et al.* (1965) found that rat liver mitochondria retained more Ca^{++} at 0 than at 30°C. However, they preloaded the mitochondria with Ca^{++} in the

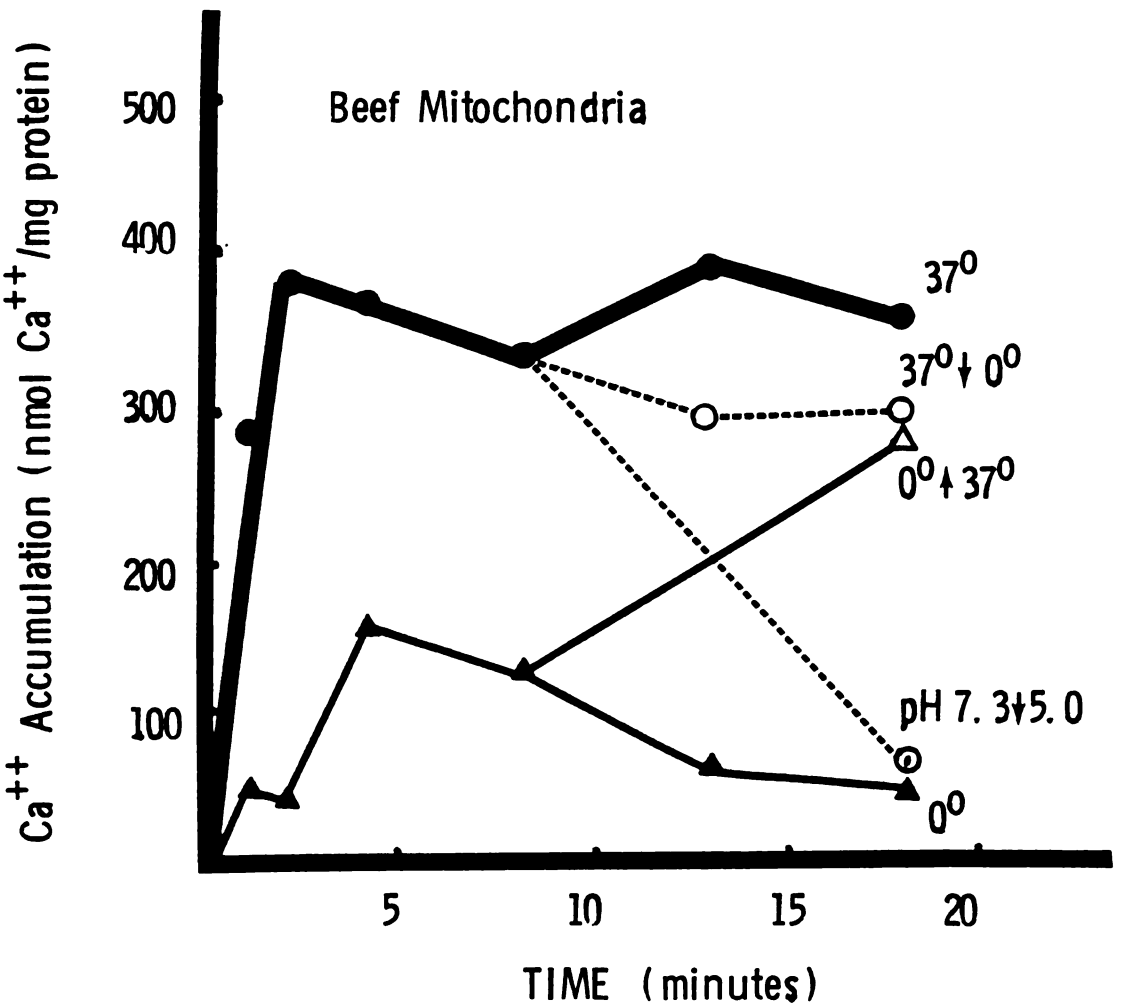

Fig. 24. Ca^{++} accumulation and release by beef mitochondria as affected by temperature and pH.

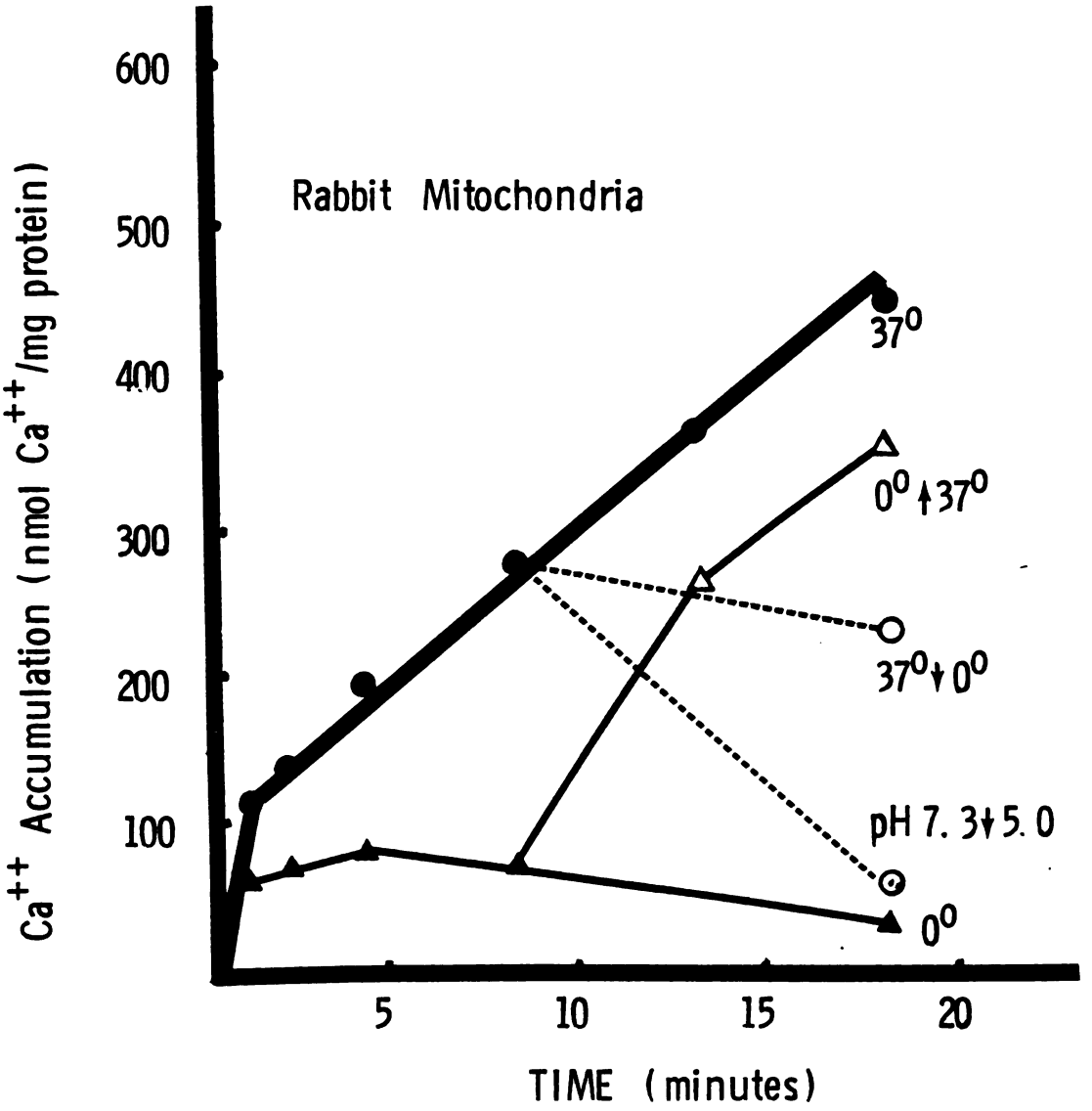

Fig. 25. Ca^{++} accumulation and release by rabbit mitochondria as affected by temperature and pH.

presence of P_i, and then resuspended the mitochondria in a buffered medium lacking ATP or respiratory substrate. Slow Ca^{++} release would be expected under such conditions. Chilling the medium would slow the rate at which Ca^{++} was released from the phosphate precipitate in the mitochondria, thus explaining their results.

The results of the present study show that chilled mitochondria release sufficient Ca^{++} to initiate cold shortening, as was previously shown for anoxic mitochondria and chilled SR preparations. These results further show that anoxic conditions are not a prerequisite for Ca^{++} release from mitochondrial suspensions, at least under in vitro conditions. Since both low temperatures and anoxic conditions are favorable for the development of cold shortening, it is probable that both conditions may contribute to the initiation of this phenomenon.

Influence of pH on Mitochondrial Ca^{++} Accumulation

Tables 6 and 7 show that both beef and rabbit mitochondria have a maximum Ca^{++} accumulating ability at pH 7.3 and 37°C, exceeding 390nmol Ca^{++} mg/protein. At pH 6.8, both preparations accumulated in excess of 230nmol Ca^{++}/mg protein, and at pH 6.2 still retain a large capacity for Ca^{++} accumulation with values in excess of 160nmol. At pH 5.5 or 5.0, however, neither mitochondrial suspensions could accumulate more than 95nmol Ca^{++}, and in general, Ca^{++} accumulation was in the range of 20-30nmol Ca^{++}/mg protein. These results

show that low pH values, in the range 5.0 to 5.5, greatly reduce the Ca^{++} accumulating ability of mitochondrial preparations, similar to the effects of low pH on SR preparations, which was previously shown herein.

Influence of pH on Mitochondrial Ca^{++} Release

Figures 24 and 25 show that beef and rabbit muscle mitochondria release almost all of the initial Ca^{++} load when the pH of the medium is lowered to pH 5.0. Both mitochondrial preparations released more than 200 nmol Ca^{++} mg protein/10 minutes under these conditions. These results suggest that the low pH encountered in postmortem muscle is sufficient to cause release of Ca^{++} from mitochondria, as well as SR membranes, and could be a factor in rigor shortening.

Effects of pH on Ca^{++} Accumulation by Mitochondria and Sarcoplasmic Reticulum

Mitochondrial and SR fractions of beef and rabbit muscle have much greater capacities for Ca^{++} accumulation at pH values of 7.3 to 6.8 than at lower values (Fig. 26). At pH 6.2, mitochondrial preparations have a significantly greater capacity for Ca^{++} accumulation than is the case for SR preparations. This suggests that muscles containing high levels of mitochondria may be more resistant to the inhibiting effects of low pH on Ca^{++} accumulation and retention, and consequently may be more resistant to the development of rigor. It is well known that the time course of rigor development is much faster in white than in red

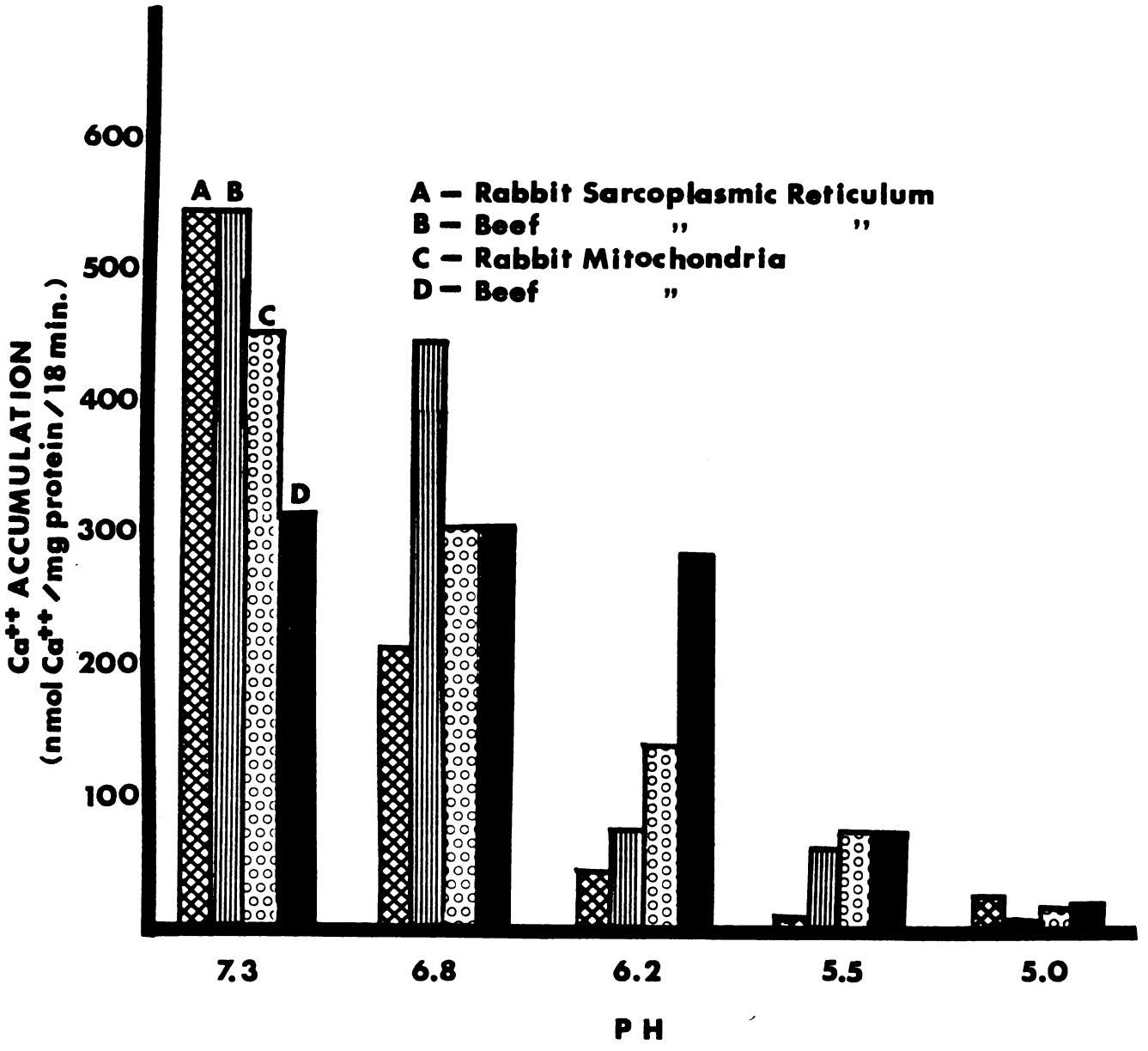

Fig. 26. Effect of pH on the Ca^{++} accumulating ability of sarcoplasmic reticulum vesicles and mitochondria of beef and rabbit muscle at $37^{o}C$.

muscles (Kastenschmidt, 1970). This has been attributed to the higher rate of postmorten glycolysis in white muscles, resulting in a faster drop in postmortem muscle pH to values at which rigor occurs (Kastenschmidt, 1970). The rate of postmortem glycolysis is undoubtedly an important factor in determining the time at which the muscle goes into rigor. Results of this study indicate that muscle mitochondria are more resistant to the effects of low pH (in the range of 6.2) than the SR membranes, which may be a contributing factor in the resistance of red muscles to rigor development. However, at pH values in the range of 5.5 to 5.0, both mitochondrial and SR membranes are inactivated with respect to Ca^{++} accumulation, and rigor ensues. These results suggest that muscle pH is important as a permissive, but not a causative factor for cold shortening. At pH values of 6.2, 6.8, or 7.3 red muscles retain the ability to accumulate Ca^{++}. If Ca^{++} accumulation is temperature sensitive, cold shortening could occur. As the postmortem muscle pH is progressively lowered, the temperature sensitivity of the muscle diminishes as observed by Locker and Hagyard (1963). Then as the pH drops to 5.5 to 5.0, Ca^{++} accumulation is markedly reduced and rigor develops. As a consequence the muscle is no longer temperature sensitive.

Effects of Temperature on Ca^{++} Accumulation by Mitochondria and Sarcoplasmic Reticulum

Figure 27 shows that mitochondria and SR preparations both have a very significant capacity for Ca^{++} accumulation

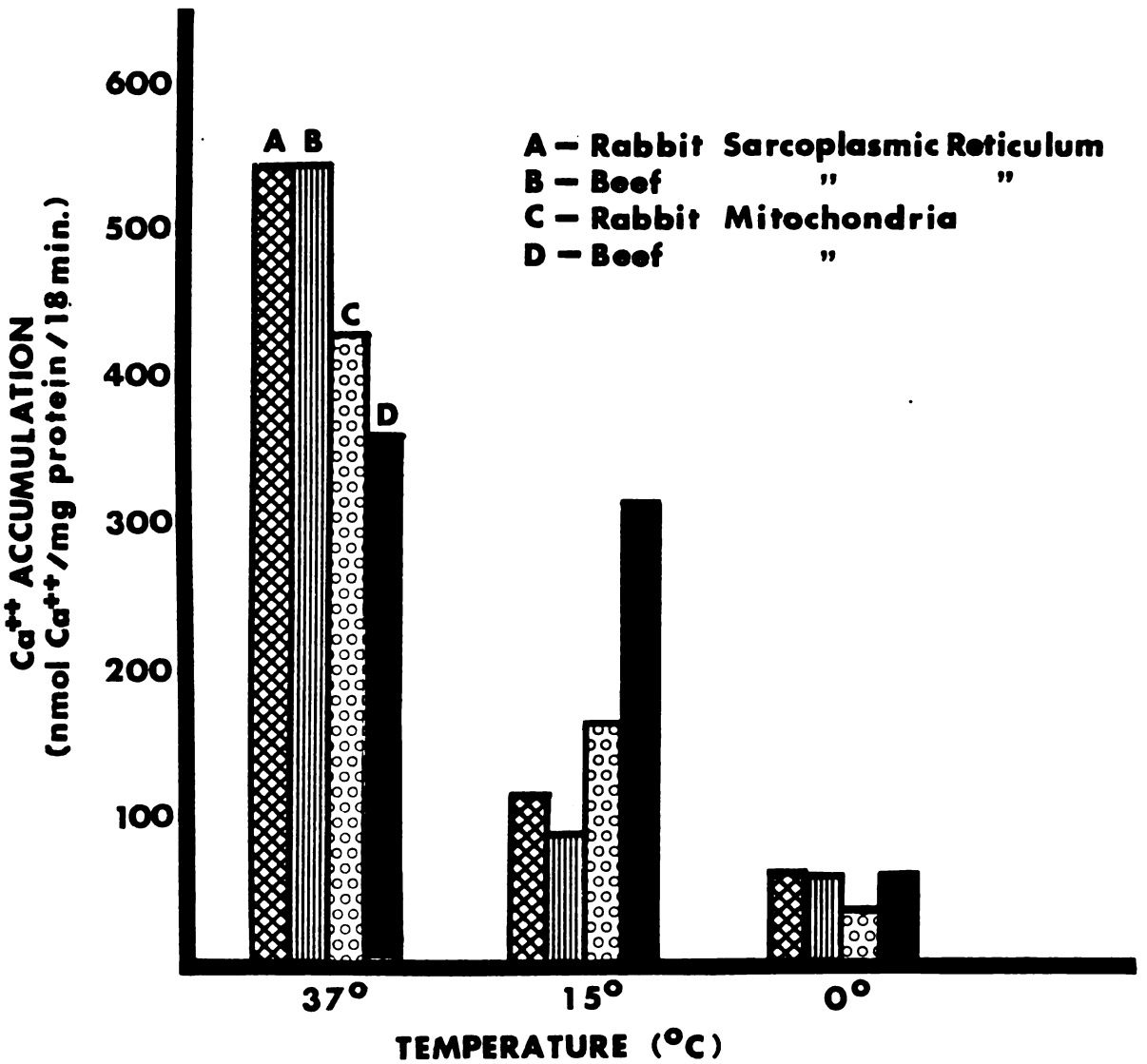

Fig. 27. Effect of temperature on the Ca^{++} accumulating ability of sarcoplasmic reticulum vesicles and mitochondria of beef and rabbit muscle at pH 7.3.

at 37°C. At 15°C both preparations retain the ability to accumulate large quantities of Ca^{++}, in the range of 100 nmol Ca^{++}/mg protein or more. However, the mitochondrial preparations have a significantly greater capacity for Ca^{++} accumulation than the SR, usually in the range of 200nmol Ca^{++} or more. The ability of the mitochondrial membranes to accumulate large amounts of Ca^{++} at 15°C may contribute to the ability of muscle to avoid cold shortening at this temperature. At 0°C both mitochondrial and SR fractions retain only a very limited ability to accumulate Ca^{++}. At this temperature, Ca^{++} is probably bound only to external sites on the membrane, in an energy or respiration independent manner (Martonosi, 1972; Lehninger et al., 1967).

These results suggest that at the temperatures which favor development of cold shortening, the ability of both SR and mitochondria to accumulate Ca^{++} is greatly reduced. Very low Ca^{++} accumulation, in combination with the release of Ca^{++} from both SR and mitochondrial sites appears to provide sufficient Ca^{++} in the myofibrillar region of the muscle to initiate cold shortening.

Reversibility of Cold Shortening

As pointed out earlier, Locker and Hagyard (1963) first observed that cold shortened muscle relaxed when returned to room temperature. However, the muscle was capable of cold shortening again, if it was chilled to 0°C. The speed and degree of cold shortening decreased with time

postmortem. After the muscle passed into rigor, it was no longer temperature sensitive.

Buege and Marsh (1975) proposed that cold shortening was the result of Ca^{++} released by anoxic mitochondria. However, their proposal offers no explanation for the repeated ability of muscle to cold shorten after being relaxed by warming, i.e., the phenomenon of reversibility (Locker and Hagyard, 1963). It is unlikely that rewarming the muscle permits respiration dependent accumulation of Ca^{++} by already anoxic mitochondria. It is even more unlikely that the anoxic mitochondria would be the direct source of Ca^{++} initiating cold shortening when the muscle is chilled a second time. On this basis, it is evident that anoxic conditions alone cannot account for the cold shortening phenomenon.

It is proposed that cold shortening results from the release of Ca^{++} as a consequence of chilling the SR, the mitochondria or both. In support of this conclusion, the present study demonstrated that SR and mitochondrial preparations from both beef and rabbit muscle release Ca^{++} as a direct result of chilling. Moreover, SR and mitochondrial preparations from both beef and rabbit muscles were capable of accumulating Ca^{++} upon warming, as would be necessary for relaxation of cold shortened muscle.

The results of this study further suggest that it is the postmortem drop in muscle pH that is responsible for the diminished reversibility of cold shortening with

increasing time postmortem. Although decreasing ATP levels and postmortem proteolysis could conceivably contribute to the inactivation of Ca^{++} accumulation by the SR membranes in postmortem muscle (Goll et al., 1971), the present study clearly shows that low pH alone will inactivate Ca^{++} accumulation by SR and mitochondrial membranes, leading to rigor.

An Explanation of Cold Shortening

The results of the present study show that isolated membrane fractions from red muscle respond in essentially the same manner and same degree to temperature, pH and anoxic conditions as those of white muscle, yet the intact muscles respond very differently to cold. Apparently, the major difference between red and white muscle is in the relative contents of the mitochondrial and SR membranes, and this difference somehow leads to cold shortening in red muscles.

Based on these observations, the following sequence of events is proposed to lead to cold shortening. After slaughter, the muscle rapidly becomes anoxic, and the mitochondria will release Ca^{++} under anoxic conditions as proposed by Buege and Marsh (1975). Both red and white muscle mitochondria release Ca^{++} under anoxic conditions, but a greater total quantity of Ca^{++} is released in red muscle, simply because red muscles contain more mitochondria. All of the Ca^{++} is accumulated by the SR membranes at room temperature, and no contraction occurs. However, the SR membranes of red muscle contain much higher concentrations of accumulated

Ca^{++}at room temperature, due to the greater amounts of Ca^{++} released, and the smaller amount of SR membranes available to accumulate Ca^{++}. When the muscles are chilled to $0^{o}C$, Ca^{++} accumulation in both muscles is greatly reduced. However, the SR of red muscles is more susceptible to the effects of chilling, due to the high concentration of accumulated Ca^{++} present, and consequently releases greater quantities of Ca^{++} than that of white muscle, resulting in cold shortening of the red muscle. This is supported by the observation of Martonosi and Feretos (1964), who found that when the Ca^{++} accumulating ability of rat SR vesicles was inhibited, the subsequent release of Ca^{++} was much faster from vesicles having higher initial Ca^{++} concentrations.

Upon rewarming the muscles, the SR membrane reaccumulates the Ca^{++}, and if sufficient ATP is present, the muscle relaxes. Chilling the muscle a second time will again lead to greater release of Ca^{++} from the red muscle SR membranes by the same process, and the muscle again shortens. As the pH drops with increasing time postmortem, the SR membranes gradually lose their ability to reaccumulate Ca^{++}, and consequently the ability to relax diminishes. When the muscle enters rigor, the SR membrane can no longer reaccumulate Ca^{++} upon warming, and the muscle is therefore temperature insensitive.

Results of the present study suggested that the mitochondria may also release Ca^{++} when chilled, and reaccumulate Ca^{++} upon warming. However, for this process to occur

in intact muscle, Ca^{++} accumulation would have to be supported by a respiration independent process, since the muscle is anaerobic. Isolated mitochondria have the capacity to accumulate Ca^{++} by a respiration independent process, i.e., ATP hydrolysis. It is unlikely, however, that the control mechanisms of intact mitochondria would permit the use of ATP for support of Ca^{++} accumulation. It is proposed, therefore, that mitochondria play no role in cold shortening other than providing the initial Ca^{++} to overload the SR membrane system of red muscle.

According to this proposal, anoxic conditions may be thought of as the priming factor, leading to release of Ca^{++} from the mitochondria, and the resultant overloading of the SR membranes in red muscle with Ca^{++}. Anoxic conditions alone are not sufficient to cause shortening, because the SR membranes can accumulate the Ca^{++} at room temperature. Chilling the muscle to $0^{o}C$ may be thought of as the initiating factor causing the release of Ca^{++} from the overloaded SR of red muscle, causing shortening. Chilling the muscle is the direct stimulus for cold shortening, and cold shortening may be reversed simply by removal of the stimulus. The muscle pH and possibly the muscle ATP levels may be thought of as permissive factors for cold shortening. At sufficiently high pH values and in the presence of adequate ATP, cold shortening may occur if the other conditions such as anoxia and chilling, exist. However, cold shortening is not permitted if the muscle pH and/or the muscle ATP levels

fall too low.

Stated somewhat differently, high mitochondrial content, anoxic conditions, high ATP levels and high pH values are all necessary for cold shortening to occur, but are not sufficient to initiate cold shortening, unless the muscle is also chilled. This is in contrast to micro injections of Ca^{++}, which overloads the SR and produces shortening even in the absence of cold (Pearson et al., 1973).

This proposal is similar to that of Buege and Marsh (1975), in that cold shortening is related to mitochondrial content of the muscle, and anoxic conditions ultimately lead to cold shortening. However, the present proposal further explains the phenomenon of reversibility of cold shortening, based on the response of the SR membrane to changes in temperature. The present study concurs with the previous conclusions of Pearson et al. (1973) and Davey and Gilbert (1974) that Ca^{++} release from SR membranes provides a source of Ca^{++} sufficient to initiate cold shortening.

SUMMARY

Ca^{++} accumulation and release by isolated SR and mitochondria of beef and rabbit muscle were determined at several pH values and temperatures. Isolation of SR and mitochondria was accomplished by homogenization of the muscle followed by differential centrifugation. Further purification of the SR was achieved by sucrose density gradient centrifugation

The yield of SR from rabbit muscle was approximately 3-fold greater than that from beef, while the yield of mitochondria from the two muscles was similar. Histochemical staining for NADH-tetrazolium reductase activity showed that beef muscle contained a much higher concentration of mitochondria. Transmission electron microscopy and SDS gel electrophoresis revealed that the SR preparations were essentially free from contamination with myofibrillar proteins or other subcellular organelles. Electron microscopy demonstrated that the mitochondrial preparations were relatively pure. Mitochondrial succinate oxidase activity was similar in suspensions from beef and rabbit muscle, but manometric measurement of oxygen consumption showed that the preparations from beef muscle contained more intact respiring mitochondria.

The SR vesicles from both beef and rabbit muscle accumulated more than 500nmol Ca^{++}/mg protein at pH 7.3 and 37°C. Mitochondria from both muscles accumulated more than 400 nmol Ca^{++}/mg protein under similar conditions. Ca^{++} accumulation by mitochondria and SR from both beef and rabbit muscle was markedly temperature dependent. At 15°C and pH 7.3, Ca^{++} accumulation was reduced, although the mitochondrial preparations still accumulated in excess of 150nmol Ca^{++}/mg protein. At 0°C, Ca^{++} accumulation by both SR and mitochondria was reduced to below 85 nmol Ca^{++}/mg protein. It is probable that at 0°C most of the Ca^{++} was bound to the external side of the membranes in an energy independent process. In spite of the reduction in Ca^{++} accumulation at low temperatures, chilled SR and mitochondria still accumulated significant quantities of Ca^{++} upon warming to 37°C.

Values for pH in the range 5.0 to 5.5 greatly reduced the capacity of SR and mitochondria to accumulate Ca^{++}. At pH 6.2, Ca^{++} accumulation by SR vesicles was low, but mitochondrial suspensions still accumulated more than 160 nmol Ca^{++}/mg protein. Ca^{++} accumulation for all preparations was maximal at pH 6.8 to 7.3

Rabbit mitochondria accumulated somewhat more Ca^{++} under anaerobic conditions than beef mitochondria. However, both rabbit and beef mitochondria accumulated significant amounts of Ca^{++} under anaerobic conditions or in the presence of the uncoupling agent, 2,4-dinitrophenol. This suggested that

at least part of the mitochondrial Ca^{++} in vitro is accumulated by a respiration-independent process, such as ATP hydrolysis. Preloaded mitochondria released small quantities of Ca^{++} when nitrogen was bubbled through the medium. Nevertheless, the quantities released were sufficient to initiate shortening in intact muscle. The data indicated that the mitochondrial preparations were sensitive to anaerobic conditions, and that mitochondrial respiration is necessary for retention of at least a portion of the accumulated Ca^{++}. Chilling of SR and mitochondrial preparations from beef and rabbit muscle also caused the release of small but significant amounts of Ca^{++}. On lowering the pH to 5.0, virtually all of the initial Ca^{++} load was released by SR and mitochondria.

Results demonstrated that SR and mitochondria from beef and rabbit muscle did not significantly differ in their response to conditions promoting cold shortening. It is, therefore, apparent that cold shortening is related to the relative concentrations of SR and mitochondria in muscle. Therefore, it is proposed that release of Ca^{++} from anoxic mitochondria leads to an overload of Ca^{++} in the SR of red muscle. The Ca^{++}, which is released upon chilling, causes shortening to occur, but may be reaccumulated upon warming. The mitochondria provide excess Ca^{++}, which ultimately leads to cold shortening, but does not play any role in Ca^{++} reaccumulation and subsequent relaxation upon warming. It is postulated that postmortem anoxia in muscles having a

high mitochondrial concentration is the priming factor leading to overloading of the SR with Ca^{++}. Application of cold is the direct stimulus or initiating factor for shortening. Sufficiently high muscle pH and ATP levels are permissive factors, since cold shortening cannot occur in their absence.

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APPENDICES

Appendix Table 1. Schedule for the preparation of 1.25% glutaraldehyde fixative solution

Reagents	Amount	Final Molarity
$NaH_2PO_4 \cdot H_2O$	1.80 g	.007
$Na_2HPO_4 \cdot 7\ H_2O$	23.25 g	.041
NaCl	5.00 g	.043
H_2O	1975.00 ml	

Appendix Table 2. Schedule for the preparation of glutaraldehyde wash buffer

Reagents	Amount	Final Molarity
$NaH_2PO_4 \cdot H_2O$	1.80 g	.013
$Na_2HPO_4 \cdot 7H_2O$	23.25 g	.081
NaCl	5.00 g	.086
H_2O	925.00 ml	

Appendix Table 3. Schedule for the preparation of Epon-Araldite resin

Reagents	Amount
Epon 813	62 ml
Araldite 506	81 ml
DDSA (hardener)	100 ml
dibutyl phthalate	4-7 ml
DMP-30	1.5-3.0%
Mixing solution:	
Epon 812, Araldite 506, and DDSA are mixed, after which the dibutyl phthalate and the DMP-30 are added. After mixing, the resin is ready for embedding the tissue.	

Appendix Table 4. Schedule for the preparation of Reynolds lead citrate stain

Reagents	Amount
Lead citrate	1.33 g
Sodium citrate	1.76 g
1 N NaOH	8 ml
H_2O (freshly boiled)	make to 50 ml

Appendix Table 5. Schedule for the preparation of a 1% osmium tetroxide fixative solution

Reagents	Amount
A. Stock Solution A	
Sodium acetate	9.714 g
Veronal-sodium	14.714 g
Make to 500 ml final volume with H_2O	
B. Stock Solution B	
Sodium chloride	40.25 g
Potassium chloride	2.10 g
Calcium chloride	.90 g
Make to 500 ml final volume with H_2O	
The solutions are mixed according to the following scheme:	
Solution A	10.0 ml
Solution B	3.4 ml
Dilute to 50 ml with H_2O	
0.1 N HCl	approx. 11 ml

Solutions A and B are measured out and made to a 50 ml volume with distilled water. The pH is then adjusted to 7.2-7.4 with 0.1 N HCl. To this mixture 0.5 g of osmium tetroxide is added and stored in a brown glass stoppered bottle.

Appendix Table 6. Schedule for the preparation of uranyl acetete stain

Reagents	Amount
Uranyl acetate	8 g
H_2O (glass distilled)	100 ml