EFFECT OF pH AND TEMPERATURE UPON Ca^{2+} RELEASE BY BEEF SARCOPLASMIC RETICULUM

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ABSTRACT

EFFECT OF pH AND TEMPERATURE UPON Ca^{2+} RELEASE BY BEEF SARCOPLASMIC RETICULUM

By

Toyoteru Kanda

The objective of this study was to investigate the effects of temperature and pH upon Ca^{2+}-release by the sarcoplasmic reticulum from beef sternomandibularis muscle in order to determine their role upon the cold shortening phenomenon. The sarcoplasmic reticulum was isolated immediately after slaughter or after holding the muscle for 24 hours at either 0 or 15°C. Isolation was accomplished by homogenization of the muscle followed by differential centrifugation, whereas, purification was achieved by sucrose density gradient centrifugation.

The yield of the sarcoplasmic reticulum was 42 $\pm$ 8 µg/gram of ground muscle for the preparation immediately post-mortem, which was only about one-tenth that from fresh rabbit muscle. On the other hand, the yield of sarcoplasmic reticulum for cold-shortened beef muscle (0°C for 24 hours) was only 25 $\pm$ 4 µg compared to a value of 16 µg per gram of ground muscle for similar muscle stored for 24 hours

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at 15°C. These results indicate that cold shortening was not responsible for the decrease in yield, but suggest that the decline in yield may be due to proteolysis of the sarcoplasmic reticulum protein.

The purity of the sarcoplasmic reticulum preparations was monitored by measuring the activity of succinate-cytochrome c reductase of the mitochondrial membrane, 5'-nucleotidase activity from the sarcolemma and the activity of acid phosphatase from the lysosomes. These tests demonstrated that the sarcoplasmic reticulum preparation was relatively pure, having only slight contamination from the mitochondria and the lysosomes and negligible contamination by the sarcolemma.

Ca^{2+} accumulation by the sarcoplasmic reticulum vesicles was determined by saturating them with $CaCl_2$ solution containing trace amounts of radioactive calcium (^{45}Ca). The release of calcium by the vesicles held under different conditions was followed by measuring the difference in the amount of accumulated calcium as monitored by radioactivity.

Sarcoplasmic reticulum vesicles from fresh (immediately post-mortem) and cold shortened (24 hours at 0°C) muscle accumulated 51 $\pm$ 2.6 and 39 $\pm$ 1.3 nM of Ca^{2+} per mg of protein, respectively, during 3 minutes at 38°C and a pH of 7.3. On the other hand, the sarcoplasmic reticulum vesicles from muscles stored for 24 hours at 15°C lost all their activity under the same conditions. The Ca^{2+} accumulating ability of fresh muscle sarcoplasmic reticulum

decreased with decreasing pH values (7.3, 6.8, 6.2, 5.5 and 5.0) at all temperatures (0, 15 and 38°C). At pH 5.0, temperature had no effect upon Ca^{2+} accumulation, with approximately 10 nM of Ca^{2+} being bound by the sarcoplasmic reticulum regardless of temperature. Maximum accumulation of Ca^{2+} (about 50 nM) occurred at 38°C and pH 7.3.

About 50% of the accumulated Ca^{2+} was released by sarcoplasmic reticulum vesicles on holding them in the reaction mixture for 10 minutes at pH 7.3 and 38°C. Changing the temperature from 38°C to 0°C at pH 6.6 resulted in the release of 20 nM of Ca^{2+} per mg of protein or a loss of 48% of the total accumulated Ca^{2+}. On the other hand, lowering the temperature from 38 to 15°C resulted in a loss of only 5 nM or about 12% of the total bound Ca^{2+} at the same pH. Thus, this study shows that low temperatures result in a much greater amount of Ca^{2+} being released from the sarcoplasmic reticulum.

The effect of simultaneously lowering the pH below 7.3 and the temperature below 38°C were much less dramatic than lowering temperature and pH independently. Approximately 10 nM of Ca^{2+} per mg of protein or about 25% of the total accumulated Ca^{2+} was released upon simutaneously lowering the pH from 7.3 to 6.6 and the temperature from 38 to 0°C. Nevertheless, results show that a simultaneous drop in pH and temperature from the physiological values (38°C and pH 7.3) can result in the release of appreciable amounts of Ca^{2+} by the sarcoplasmic reticulum.

SDS-gel electrophoresis of the purified beef sarcoplasmic reticulum gave four major protein bands including a broad diffuse band. Molecular weights of the four major proteins were estimated to be 100,000, 63,000, 58,000 and less than 10,000 daltons. The 100,000 molecular weight protein appeared to correspond to the Ca^{2+} activated ATPase, which has been identified in rabbit sarcoplasmic reticulum. Although the 63,000 and 58,000 molecular weight proteins did not have the same Rm values and molecular weights as Calsequestrin and the high affinity Ca^{2+}-binding protein, which have been found in rabbit sarcoplasmic reticulum, further work will be needed to prove whether or not they perform the same functions in beef sarcoplasmic reticulum.

Electron microscopic examination of fresh and cold shortened muscle sarcoplasmic reticulum vesicles from beef failed to reveal any differences in size or conformation. Thus, results indicate cold shortening caused no observable differences in the ultrastructure as seen by electron microscopy.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX FIGURES

INTRODUCTION

Muscle is known to undergo a large number of biochemical and physical changes post-mortem. It is well recognized that exposure of excised fresh beef muscle to temperatures near the freezing point causes appreciable shortening, which is commonly referred to as "cold shortening" (Locker and Hagyard, 1963). Newbold (1966) stated that cold shortening is complete by the time the pH has fallen to about 6.2 and the level of ATP to about 40% of its initial value, but a clear explanation is lacking as to the exact mechanism of the cold shortening phenomenon.

Marsh (1966) speculated that cold shortening was due to inactivation of the relaxing factor with the release of Ca^{2+} ions. The relaxing factor, which was originally discovered by Marsh (1951), is found in the sarcoplasmic reticulum. It has the ability to remove Ca^{2+} from solution by an ATP-dependent transport process (Ebashi *et al.*, 1962; Hasselbach *et al.*, 1961).

Since most work with fragmented sarcoplasmic reticulum has been concerned with its function in living muscle, little information is available about changes in its activity post-

mortem (Greaser et al., 1967, 1969a, 1969b; Schmidt, 1970; Goll, 1971; Hay et al., 1973). Thus, information is needed on the relationship of the Ca^{2+}-accumulating ability of the sarcoplasmic reticulum to cold shortening. Consequently, this investigation was undertaken to observe the effects of pH and temperature treatments on Ca^{2+} release from Ca^{2+} saturated purified beef sarcoplasmic reticulum.

LITERATURE REVIEW

Cold Shortening

Cold shortening was first observed by Locker and Hagyard (1963). They observed that excised and unrestrained beef muscle shortened more rapidly at 0°C than at any other temperature. Maximum shortening amounted to 47.7% and occurred at 0°C. They also observed that minimum shortening (less than 10%) occurred at 14°C to 19°C.

Although the first observations were made with beef sternomandibularis (neck) muscle, the same phenomenon was also found to occur for beef longissimus dorsi (ribeye) muscle and to a lesser extent for beef psoas major (tenderloin) muscle (Locker and Hagyard, 1963).

Rabbit pre-rigor white muscle has been shown not to shorten on exposure to cold (Locker and Hagyard, 1963; Henderson *et al.*, 1970), but rabbit red muscle (semitendinosus) shortens appreciably under cold conditions (Bendall, 1966). Porcine longissimus dorsi muscle shortens slightly more at 2°C than at 25°C, but not as much as at 37°C (Henderson *et al.*, 1970). The cold shortening effect was also observed to occur in lamb muscle (Marsh *et al.*, 1968).

Smith et al. (1969) found that excised pectoralis major muscles of chicken and turkey shortened significantly more at 0°C than at 12-18°C, but Jungk and Marion (1970) detected no cold shortening in turkey pectoralis held at 4°C.

A relationship between cold shortening and the degree of meat tenderness has been shown to exist (Marsh et al., 1966, 1972; Jungk and Marion, 1970). Marsh (1968) stated that significant toughness developed in the longissimus dorsi muscle of lamb carcasses exposed to low temperatures during the first 16 hours following slaughter. Marsh et al. (1966) also found that less than 20% shortening in steromandibularis muscle from beef caused little or no toughening, but 20% to roughly 40% shortening caused maximum toughness. Beyond 40% shortening the meat rapidly became more tender.

Role of the Sarcoplasmic Reticulum in Regulation of Ca^{2+} Concentration

Marsh (1951, 1952) observed that the volume of the sedimented crude myofibrillar fraction remained relatively large in the presence of Mg^{2+} and ATP, while addition of a small amount of Ca^{2+} resulted in sudden shrinkage. Crude myofibrils resuspended in 0.1 M KCl upon addition of 1 mM ATP underwent immediate shrinkage, even in the absence of added Ca^{2+} (Marsh, 1951, 1952). On the basis of these experiments, Marsh (1951, 1952) suggested that a substance in the muscle extract was intimately involved

in the myofibrillar volume changes. He called this substance "relaxing factor," i.e., fragmented sarcoplasmic reticulum. Marsh (1952) suggested that close packing and swelling of the myofibrillar sediment corresponded to contraction and relaxation, respectively, and then concluded that the relaxing factor was responsible for regulating muscle contraction and relaxation.

The observations of Marsh (1951, 1952) were soon extended by Bendall (1952, 1953) and by Hasselbach and Weber (1953), who found that glycerol-extracted muscle fibers, which had contracted under the influence of ATP in the presence of Mg^{2+}, immediately became relaxed upon the addition of the so-called "Marsh factor." A number of researchers (Kumagai et al., 1955; Lorand et al., 1957; Portzehl, 1957a, b) have tried to isolate the factor causing relaxation of muscle fibrils. Electron microscopic evidence has shown that the substance having relaxing activity is located in the sarcoplasmic reticulum (Muscatello et al., 1961; Nagai et al., 1960; Ebashi and Lipmann, 1962).

Ebashi (1960, 1961a, b) and Ebashi and Lipmann (1962) discovered that sarcoplasmic reticulum fragments in the presence of ATP and Mg^{2+} actively remove Ca^{2+} from the medium. Inhibition of syneresis and ATPase activity of actomyosin and myofibrils by sarcoplasmic reticulum fragments was accompanied by a reduction in their bound Ca^{2+} content (Weber et al., 1963, 1964a, b;

Ebashi, 1961a, b; Fanburg, 1964; Fanburg et al., 1964; Weber et al., 1967). The information outlined above clearly indicates that the contractile system is regulated by the concentration of free Ca^{2+} in the sarcoplasm, and that the sarcoplasmic reticulum is able to lower the free Ca^{2+} concentration to levels one would expect to find in relaxed muscle.

A hypothetical picture of events occurring during a contraction-relaxation cycle has been outlined by Martonosi (1971). During the relaxed state, the Ca^{2+} ions are stored in the sarcoplasmic reticulum, thus, the concentration of free Ca^{2+} in the sarcoplasm is low, and actin and myosin are dissociated. On excitation, a depolarization wave generated by a nerve impulse spreads through the T-system into the interior of the muscle fiber, and by an as yet unknown mechanism, triggers the release of Ca^{2+} from the sarcoplasmic reticulum into the sarcoplasm. The evaluation of the Ca^{2+} concentration in the environment of the myofilaments brings about the interaction of actin and myosin, resulting in muscle contraction. As the membrane is repolarized, the concentration of Ca^{2+} in the sarcoplasm is lowered by the Ca^{2+}-pump of the sarcoplasmic reticulum. Actomyosin is then dissociated into actin and myosin, and relaxation returns.

Several workers (Natori, 1954, 1955, 1965; Podolsky, 1962; Podolsky and Costantin, 1964; Weber, 1966; Feinstein, 1966) have shown that the internal concentration of free

Ca^{2+} during the resting state in muscle must be less than 10^{-6} M.

Mechanism of Ca^{2+} Transport

Sarcoplasmic reticulum membranes contain a Ca^{2+}- and Mg^{2+}-dependent ATPase (Hasselbach and Makinose, 1961). While accumulation of Ca^{2+} by the sarcoplasmic reticulum requires the presence of ATP and Mg^{2+} (Ebashi, 1960, 1961a, b; Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961, 1963), the energy for the Ca^{2+} transport system is supplied by hydrolysis of ATP through the action of ATPase, which is activated by Ca^{2+} in the presence of Mg^{2+}.

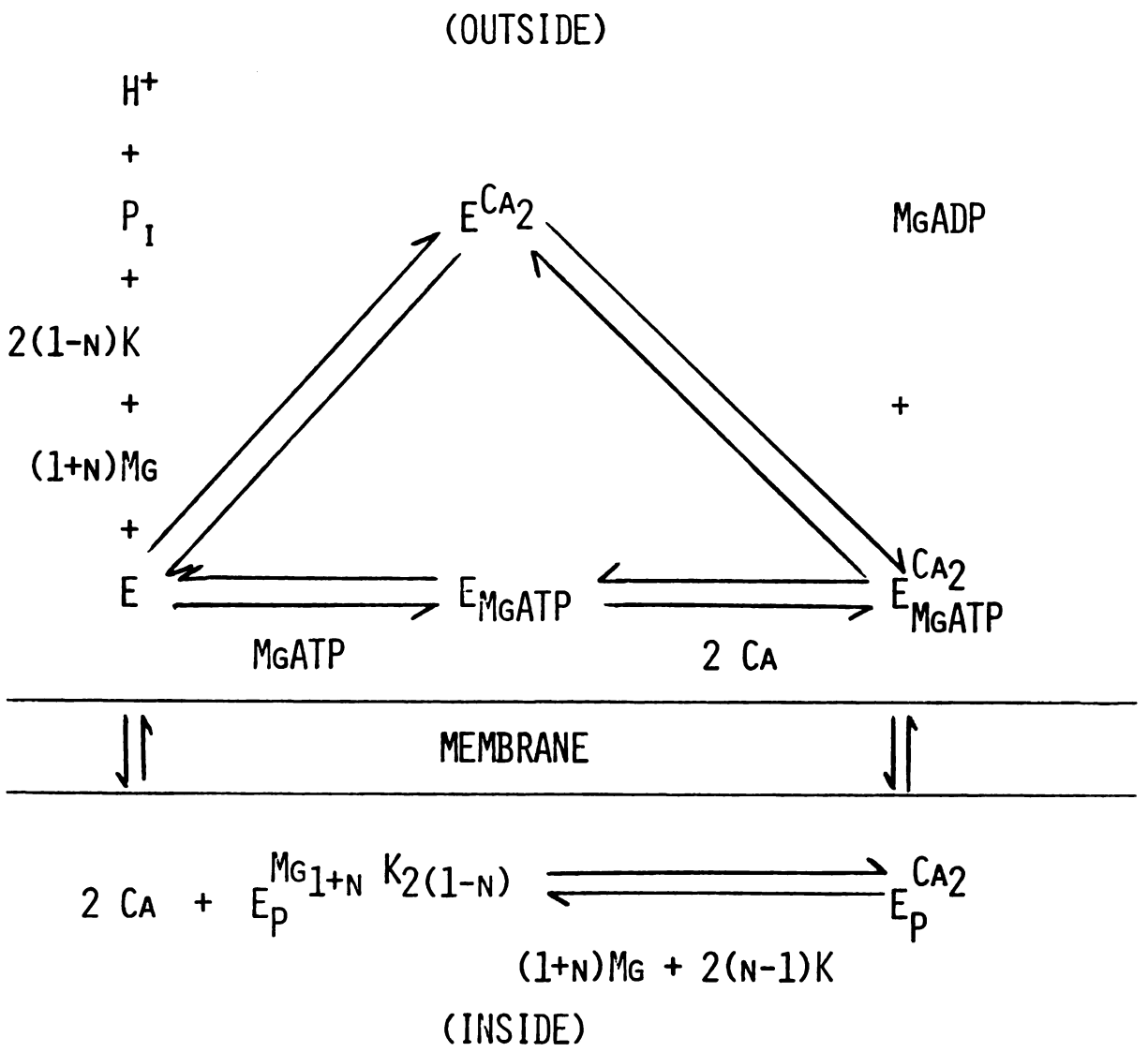
The coupling mechanism between Ca^{2+} binding and ATPase in the sarcoplasmic reticulum has been studied by many workers (Hasselbach and Makinose, 1961, 1963; Ebashi and Lipmann, 1962; Weber et al., 1966; Yamada et al., 1970; Yamada et al., 1972; Yamamoto et al., 1967). The presence of a phosphorylated intermediate in the ATPase reaction was suggested by discovery of ADP - ATP exchange activity in the sarcoplasmic reticulum (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1962, 1965). Subsequently, formation of a Ca^{2+}-dependent phosphorylated protein (EP) from the sarcoplasmic reticulum and ATP was discovered (Yamamoto et al., 1967, 1968; Makinose, 1969; Martonosi, 1969). It was found that the EP was a high-energy, phosphate-type compound (Kanazawa et al., 1971). The formation of EP + ADP from E + ATP (EP + ADP = E + ATP)

involves external Ca^{2+}. The reverse reaction, i.e., the formation of EP and ADP from E and ATP, requires the presence of internal Ca^{2+} (Kanazawa et al., 1970, 1971). In other words, EP formation is activated by Ca^{2+} outside the sarcoplasmic reticulum membrane, but the reverse reaction is activated by Ca^{2+} inside the membrane (Kanazawa et al., 1970, 1971). The reaction is shown below:

$$E + MgATP \underset{Ca^{i}}{\overset{Ca^{o}}{\rightleftharpoons}} EP + ADP + Mg^{2+}$$

The superscript i indicates the Ca^{2+} ions are inside of the membrane, while superscript o indicates the Ca^{2+} ions are located outside the membrane. These results indicated to Kanazawa et al. (1971) that the Ca^{2+} binding site is translocated from the outside to the inside of the sarcoplasmic reticulum membrane.

Kanazawa et al. (1971) also found that Mg^{2+} stimulate decomposition of EP inside of the sarcoplasmic reticulum membrane. From these results the following reaction scheme was proposed for transport of Ca^{2+} by Kanazawa et al. (1970, 1971) and Yamamoto (1968, 1969).



Ca^{2+} Accumulating Ability

The maximum amount of Ca^{2+} accumulated by sarcoplasmic reticulum vesicles of rabbit muscle in the absence of Ca^{2+} precipitating agents is in the range of 50 - 250 nM Ca^{2+}/mg protein (Ebashi and Lipmann, 1962; Ohnishi and Ebashi, 1963; Weber et al., 1964a, b; Van der Kloot and Glovsky, 1965; Ebashi et al., 1964; Harigaya et al., 1968; Sommer and Hasselbach, 1967; Sreter, 1969; Nakamaru and Schwarz, 1970; Cohen and Selinger, 1969; Ogawa, 1970; Greaser et al., 1967; MacLennan

et al., 1971). The reported values for the maximum amount of accumulated Ca^{2+} vary somewhat from preparation to preparation. Purity of the preparation, the reaction mixture (composition and pH), the method of measuring Ca^{2+} accumulation and the reaction temperature appear to be responsible for these differences.

Recently, Harigaya and Schwartz (1974) reported that fragmented sarcoplasmic reticulum from white skeletal muscle of the rabbit had the greatest Ca^{2+} accumulating ability (160 nM Ca^{2+}/mg protein), whereas, the sarcoplasmic reticulum from red skeletal muscle of the rabbit bound the second largest amount of Ca^{2+} (80 nM Ca^{2+}/mg protein). The lowest Ca^{2+} accumulating ability was found in the sarcoplasmic reticulum from rabbit cardiac muscle (40 nM Ca^{2+}/mg protein).

Ca^{2+} precipitating agents, such as oxalate, inorganic phosphate (Hasselbach and Makinose, 1961; Martonosi and Feretos, 1964) and other compounds (Lorand and Molnar, 1962, Ebashi and Endo, 1964; Martonosi and Feretos, 1964), increase the amount of Ca^{2+} accumulated by sarcoplasmic reticulum fragments. The increased Ca^{2+} uptake is due to the precipitation of a Ca^{2+} salt in interior of the vesicles. Ca^{2+} uptake of the sarcoplasmic reticulum from rabbit muscle in the presence of the Ca^{2+} precipitating agent was in the range of 0.6 - 5.0 μM Ca^{2+}/mg protein (Hasselbach and Makinose, 1962; Makinose and Feretos, 1964; Lorand and Molnar, 1962; Ebashi and Endo, 1964; Weber et al., 1966; Ebashi et al., 1964; Sommer and Hasselbach, 1967; Harigaya et al.,

1968; Meissner and Fleisher, 1971; MacLennan and Wong, 1971; Meissner et al., 1973).

Release of Ca^{2+}

Ca^{2+} ions can be reversibly released by depolarization of the sarcoplasmic reticulum (Martonosi, 1971), and also by applying drugs, such as caffeine and thymol (Weber and Herz, 1968; Johnson and Inesi, 1969; Ogawa, 1970). The release of Ca^{2+} by caffeine has been shown to be the result of direct action of the drug on the sarcoplasmic reticulum (Ebashi et al., 1969). However, addition of caffeine does not markedly inhibit the uptake of Ca^{2+} (Ogawa, 1969; Weber and Herz, 1968). According to Ogawa (1970), the Ca^{2+} releasing action of caffeine was more effective in the heavier fraction (1,200 - 7,000 x g) of the sarcoplasmic reticulum and at lower temperatures. Sakai (1965) and Sakai and Conway (1960) also found that sudden lowering of the temperature to 1 - 3°C in a muscle system pretreated with caffeine caused strong contracture.

Thymol showed essentially the same effect as that of caffeine, but was about thirty times more effective (Ogawa, 1970). EDTA and EGTA also have the same effect on release of Ca^{2+} (Duggan and Martonosi, 1970; Panet and Selinger, 1972).

Panet et al. (1972) observed that Ca^{2+} release from sarcoplasmic reticulum fragments was enhanced by the addition of low concentrations of ADP and P_i, but the effect was abolished by the presence of Mg^{2+}. It was observed

that a sudden change of the assay medium to the alkaline pH range also causes Ca^{2+} release (Duggan and Martonosi, 1970). Carvalho and Leo (1967) have shown that H^+ ions replace Ca^{2+} at the binding sites of fragmented sarcoplasmic reticulum below pH 6.2 in the presence of ATP. Bertrand et al. (1971) also observed that low pH causes a marked reduction in the affinity of the sarcotubular membranes to bind Ca^{2+}.

Mild heat denaturation also causes Ca^{2+} leakage (30 - 50°C) of the sarcoplasmic reticulum (Johnson and Inesi, 1969; Inesi et al., 1973; Hasselbach et al., 1969; Duggan and Martonosi, 1970).

Ca^{2+} Accumulation of the Sarcoplasmic Reticulum in Post-Mortem Muscle

The Ca^{2+} accumulating ability of the sarcoplasmic reticulum from porcine muscle has been shown to drop markedly with increasing post-mortem time (Greaser et al., 1967, 1969a, b; Schmidt et al., 1970). Greaser et al. (1967) stated that the heavy sarcoplasmic reticulum fraction from muscle held for 3 hours post-mortem lost about 40% of its Ca^{2+} accumulating ability, and by 24 hours post-mortem had declined to only 10% or less of the initial value. However, electron microscopic observations of each fraction showed no difference in appearance (Greaser et al., 1967).

Greaser et al. (1969a) suggested that a low muscle pH accompanied by a high carcass temperature may be responsible for the loss in the Ca^{2+} accumulating ability

of the sarcoplasmic reticulum in pig muscle. In a subsequent study, Greaser et al. (1969b) found that pH values below 6.0 reduced the ability of sarcoplasmic reticular membranes to sequester Ca^{2+}. The Ca^{2+} accumulating ability of pale, soft, exudative (PSE) porcine muscle was lost during the first hour after death, whereas, normal muscle showed a more gradual decrease (Greaser et al., 1969b).

Goll et al. (1971) observed that sarcoplasmic reticular membranes from rabbit muscle lost their ability to sequester Ca^{2+} just prior to the onset of rigor mortis. However, the ATPase activity of fragmented sarcoplasmic reticulum remained almost constant from death until maximum tension development (Goll et al., 1971). On the other hand, Nauss and Davies (1966) have shown that post-mortem tension development of frog sartorius muscles was always accompanied by an increased rate of Ca^{2+} efflux, even in the presence of ATP.

Goll et al. (1971) have suggested three possible causes for the loss in the Ca^{2+} accumulating ability of post-mortem sarcoplasmic reticular membranes: (1) uncoupling of the Ca^{2+} pump by proteolysis; (2) the post-mortem pH decline; and (3) the post-mortem loss of ATP. However, they concluded that proteolysis is the principal factor responsible for the loss of the Ca^{2+} accumulating ability in post-mortem muscle.

An increase in the Ca^{2+} accumulating ability of sarcoplasmic reticulum fragments from chicken breast muscle

following post-mortem aging was reported by Hay et al. (1973). They suggested that the increase in Ca^{2+} accumulating capacity may be due to a greater concentration of Ca^{2+} sequestering sarcoplasmic reticulum fragments. Hay et al. (1973) also suggested that the increased lipid content of sarcoplasmic reticulum in aged muscle may increase Ca^{2+} accumulation.

Protein Components

Several proteins have been found in rabbit sarcoplasmic reticulum, most of which interact strongly with Ca^{2+} (Martonosi, 1969; Martonosi and Halpin, 1971; MacLennan, 1970, 1974; MacLennan and Wong, 1971; MacLennan et al., 1971, 1972; Ikemoto et al., 1972; Meissner et al., 1973; Inesi and Scalers, 1974; Ostwald and MacLennan, 1974a, b).

The major protein in the sarcoplasmic reticulum is ATPase, and estimates of its percentage of the total sarcoplasmic reticular proteins vary between 18 and 90% (Martonosi, 1968; Salinger and Klein, 1969; MacLennan, 1970; McFarland and Inesi, 1970; Martonosi and Halpin, 1971; MacLennan et al., 1971; Meissner and Fleischer, 1971). However, recent reports have suggested that the true ATPase content of the sarcoplasmic reticulum is approximately 70% of the total protein (Meissner et al., 1973; Inesi and Scalers, 1974). Most studies have shown that ATPase has a molecular weight of approximately 100,000 (Martonosi, 1968; Salinger and Klein, 1969; MacLennan, 1970; Inesi et al., 1970; McFarland and Inesi, 1970; Martonosi and Halpin,

1971; MacLennan and Seeman, 1971; Meissner and Fleisher, 1971). ATPase, which is water-insoluble, requires phospholipid, Mg^{2+} and Ca^{2+} for activity, reacts with phospholipids to form membrane vesicles (Stewart and MacLennan, 1974), and is a globular protein extending through the sarcoplasmic reticular membrane (MacLennan et al., 1972).

The next most predominant protein in the sarcoplasmic reticulum is "Calsequestrin," which was isolated and named by MacLennan and Wong (1971). This protein has also been called the 55,000 dalton protein (Ikemoto, 1972; Ostwald and MacLennan, 1973).

Calsequestrin is an extremely acidic protein with 37% of the total amino acid residues being acidic, of which less than 10% are amidated, while only 8% are basic (Ostwald and MacLennan, 1974; Meissner et al., 1973). The protein binds large quantities of Ca^{2+} (35 - 43 moles per mole) at pH 7.5, with an apparent dissociation constant of 40 - 60 μM in the absence of KCl (Stewart and MacLennan, 1974). This unique protein of the sarcoplasmic reticulum is hydrophobically bonded on the interior of the membrane, and is believed to play a role in sequestering Ca^{2+} within the membrane (MacLennan and Wong, 1971).

MacLennan (1974) recently isolated a second form of Calsequestrin, which was found to have a molecular weight (Form 2, 43,700) 6% less than that of the previously reported Calsequestrin (Form 1, molecular weight 46,500). He also found that Form 1 and 2 were similar in amino acid

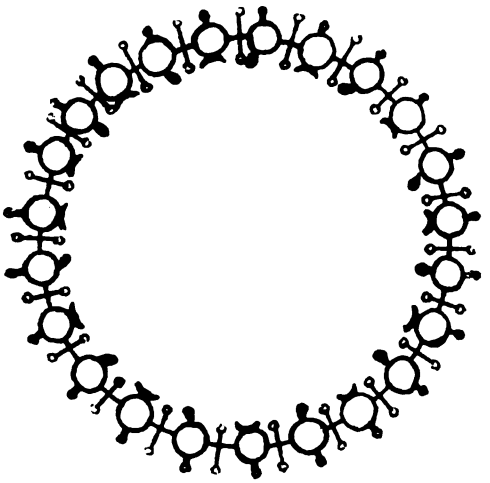
composition, with the major difference being in the content of tyrosine, cysteine and methionine.

The third protein found in the sarcoplasmic reticulum is the high affinity Ca^{2+} binding protein (molecular weight 55,000), but it has only one half of the total Ca^{2+} binding capacity of Calsequestrin (Ostwald and MacLennan, 1974). The difference in Ca^{2+} binding capacity may be due to the amino acid composition of the two proteins; Calsequestrin has 37% acidic amino acid residues and only 8% of basic amino acids, whereas, the high affinity Ca^{2+} binding protein has 32% acidic amino acids and 13% basic amino acids (Ostwald and MacLennan, 1974). Calsequestrin and the high affinity Ca^{2+} binding protein each account for 5 - 10% of the total sarcoplasmic reticulum protein (Meissner et al., 1973; MacLennan and Wong, 1971).

A group of acidic proteins with molecular weights between 20,000 and 38,000 was also isolated from the sarcoplasmic reticulum by MacLennan et al. (1972, 1974). These proteins bind large amounts of Ca^{2+} with low affinity (Stewart and MacLennan, 1974). Upon SDS gel electrophoresis of the sarcoplasmic reticulum, an opalescent band was found in oblique light and identified as proteolipid by MacLennan et al. (1972). They found that it had a molecular weight of 6,000 daltons.

Thus, as described above, seven proteins have been isolated from the sarcoplasmic reticulum by MacLennan et al. (1971, 1972, 1974). They have suggested a hypothetical

scheme for the structural arrangement of these proteins with the lipid in vesicles of the sarcoplasmic reticulum as shown in the diagram below (MacLennan et al., 1972):



ATPase, a phospholipid bilayer and a proteolipid make up the membrane continum. The proteolipid may act as a bimodal molecule orienting the hydrophilic ATPase within the hydrophilic phospholipid bilayer. Calsequestrin and the 55,000 molecular weight protein (the high affinity Ca^{2+} binding protein) are localized on the interior surface of the sarcoplasmic reticular membrane and are believed to play an important role in binding and sequestering the high concentration of Ca^{2+} on the interior of the sarcoplasmic reticulum vesicles.

MATERIALS AND METHODS

Preparation of the Sarcoplasmic Reticulum

Sternomandibularis (neck) muscles from beef carcasses slaughtered in the Michigan State University abattoir were used in this study. The muscles were excised from the carcasses immediately following slaughter. All external fat and connective tissue were dissected from the muscle samples prior to use. The sarcoplasmic reticulum was isolated from the muscles immediately after trimming, or else the trimmed muscles were stored for 24 hours at either 15 or 0°C prior to isolation of the sarcoplasmic reticulum.

Sarcoplasmic reticulum vesicles were prepared from all muscles using the procedures of Meissner and Fleisher (1971). Figure 1 shows the procedure followed in isolation of the sarcoplasmic reticulum vesicles. All steps were carried out at 0°C. The muscle was passed through a meat grinder. A total of 200 grams of ground muscle was homogenized in 4 volumes of homogenization buffer consisting of 0.3 M sucrose and 10 mM N-2-hydroxyethylpiperazine-n'-1-ethansulfonic acid (HEPES) buffer (pH 7.4) for 30 sec in a

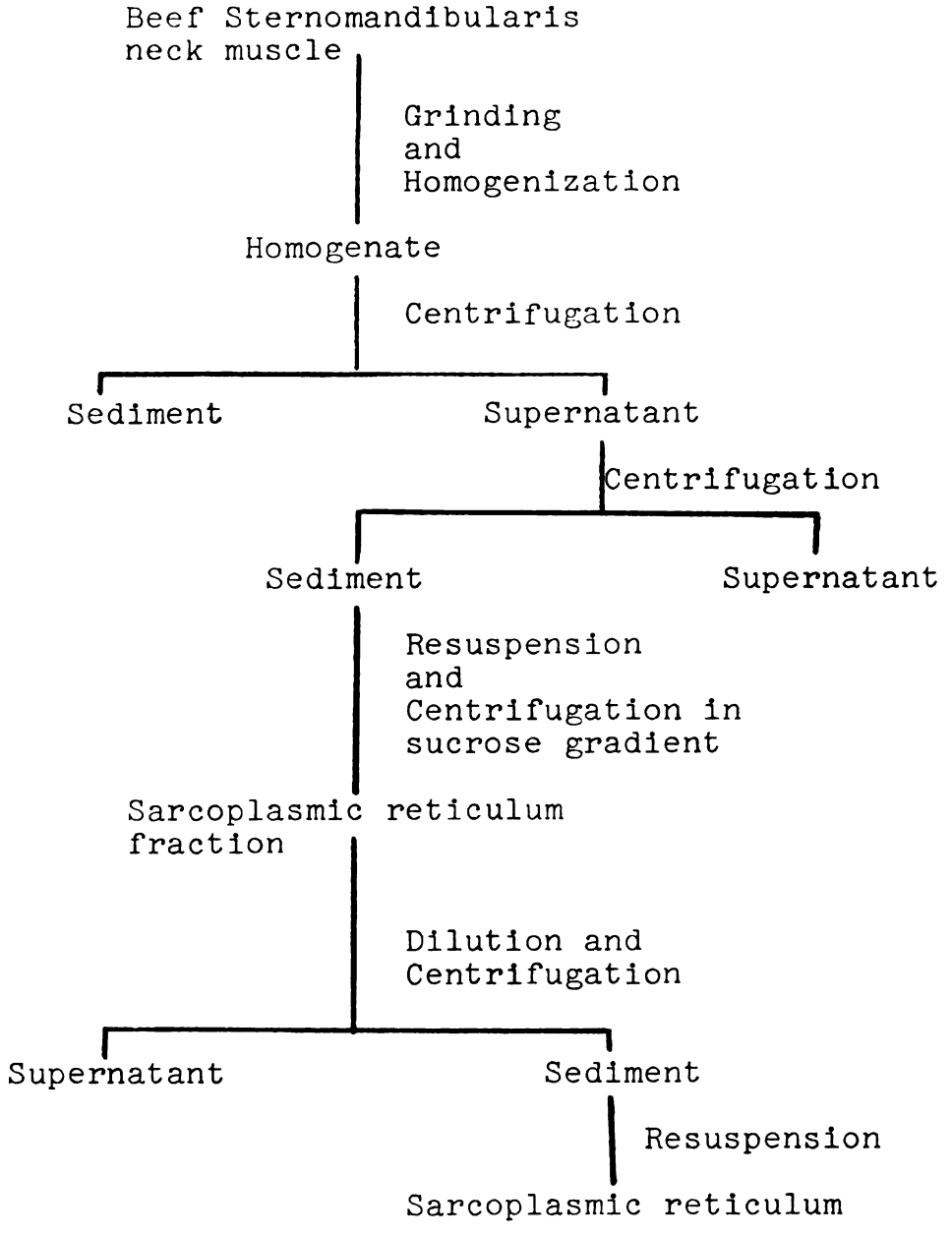


Figure 1. Flow sheet of procedure used in isolation of the sarcoplasmic reticulum.

Waring blendor. The homogenate was centrifuged in a Sorvall centrifuge (Model RC2-B, Sorvall, Inc.) for 20 min at 7000 rpm using a GSA rotor. The supernatant was strained through eight layers of cheese cloth, and a crude fraction of sarcoplasmic reticulum vesicles was obtained by centrifugation for 75 min at 28,000 rpm (85,500 X g) in a A-170 rotor of an IEC preparative ultracentrifuge, Model B-60 (International Equipment Company, Needham Heights, Massachusetts). The supernatant was poured off, and the pellet was resuspended in a total volume of 18 ml of the homogenization buffer using a Polytron homogenizer (Kinematica., Luzern-Schweiz).

The crude sarcoplasmic reticulum was placed on top of a discontinuous gradient containing 5 mM HEPES buffer (pH 7.4) with different percentages of sucrose in each layer as shown in Figure 2. The sucrose concentration in percent (w/w) was adjusted using a Valentine Refractometer (Valentine and Company, Vista, California). After application of 3 ml of crude sarcoplasmic reticulum, the tubes were spun for 2.5 hours at 23,500 rpm in SB-283 rotor in an IEC preparative ultracentrifuge.

Vesicle fractions were carefully removed from the gradient with a pipette. The top 4.8 ml of the gradient were discarded, and the next 3.9 ml fraction was collected for further studies (Figure 2). The fraction was then diluted with 2 volumes of 5 mM HEPES buffer (pH 7.4) added in four equal parts over a period of 30 - 45 min in order to minimize osmotic shock. It was then centrifuged for

layer No.	% sucrose (w/w)	volume (ml)	fraction (ml)
sample	9.8	3.0	4.8
1	26.4	2.4	
2	29.1	1.6	3.9 (sarcoplasmic reticulum fraction)
3	31.6	1.6	
4	33.9	2.0	
5	37.2	2.2	

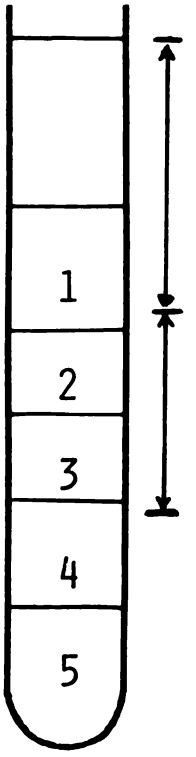


Figure 2. Fractionation of the sarcoplasmic reticulum on a sucrose step gradient.

1 hour at 35,000 rpm in the SB-283 rotor in the IEC preparative ultracentrifuge.

The pellets were resuspended in a solution containing 0.3 M sucrose and 2.5 mM HEPES buffer (pH 7.4) and stored at 0°C until used. Some of the resuspended sarcoplasmic reticulum was frozen using dry ice - acetone and stored at -20°C for stability studies on isolated sarcoplasmic reticulum. Ultrapure sucrose (Schwarz-Mann, Orangeburg, New York) was used throughout the experiments.

pH Measurements

A portion of the fresh trimmed muscle was stored at 0°C for pH measurements on the sarcoplasmic reticulum. Small portions were removed after storage for 0, 0.5, 1, 3, 5, 10 and 24 hours. The samples were removed and homogenized in 5 volumes of distilled-deionized water and then the pH of the homogenate was measured using an expanded scale pH meter (Radiometer Copenhagen, Type PHM 26).

Ca^{2+} Accumulation and Ca^{2+} Release Determination

Reaction mixture.

The Ca^{2+} transport system requires ATP, Mg^{2+} and Ca^{2+} for activation. The Ca^{2+} accumulation and Ca^{2+} release were determined using a reaction mixture containing 100 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 10 mM histidine and 0.1 mM $CaCl_2$. The reaction mixture was adjusted to pH 7.3. $^{45}CaCl_2$ was added to give a final count of 80,000 cpm per

ml in the reaction mixture. The $^{45}CaCl_2$ was obtained in aqueous solution from Amersham-Searle Corp. (Arlington Heights, Illinois).

pH adjustment of the reaction mixture.

The pH of the reaction mixture was adjusted by addition of 0.1 N HCl in order to give solutions with pH value of 6.6, 6.2, 5.8, 5.6 and 5.0 for measurement of Ca^{2+} accumulation.

Determination of Ca^{2+} accumulation.

Three ml of the reaction mixture were placed in a tube, and if necessary, 0.1 N HCl was added to a 100 µl microsyringe to give the desired pH. Then the reaction mixture was equilibrated to 38, 15 or 0°C. The reaction was initiated by addition of 40 - 80 µg of sarcoplasmic reticulum protein per ml of reaction mixture. The reaction was carried out for 3 min and terminated by filtration through a Millipore filter, type GS, average pore size 0.22 µ (Martonosi and Feretos, 1964). Ca^{2+} accumulation was calculated from the difference in radioactivity between the reaction mixture without the added sarcoplasmic reticulum (control) and that containing added sarcoplasmic reticulum filtrate prepared as described earlier.

Determination of Ca^{2+} release.

Two tubes containing 3 ml of the reaction mixture were equilibrated at 38°C. The reaction was initiated by the addition of 40 - 80 µg/ml of sarcoplasmic reticulum

protein and continued for 3 min at 38°C. One of the tubes was filtered through a Millipore filter as described earlier. Another tube was transferred from 38°C to either a 15 or 0°C constant temperature water bath in order to lower the temperature of the reaction mixture, and was then incubated for 10 min. In some experiments, the pH of reaction mixture was changed by rapid addition of 0.1 N HCl to the desired value before transferring the tube to constant temperature water bath and incubating for 10 min. After incubation, the reaction mixture was passed through a Millipore filter.

Ca^{2+} release was calculated from the amount of accumulated Ca^{2+} in the sarcoplasmic reticulum vesicles before and after the pH and/or temperature of the reaction mixture was changed.

Determination of radioactivity.

Radioactivity of $^{45}Ca^{2+}$ was determined by counting aliquots of filtrates which were mixed with PCS, a scintillation liquid (Amersham-Searle Corp., Arlington Heights, Illinois). Either a Packard model 3310, TRI-CARB scintillation spectrometer (Packard Instrument Company Inc., Illinois) or Nuclear-Chicago, Mark 1, model 6894, a liquid scintillation counter, (Amersham-Searle Corp., Arlington Heights, Illinois) was used for counting radioactivity. The following counting conditions were used: (1) Packard TRI-CARB scintillation spectrometer: window setting - 50 - 1,000, and gain - 11.5%; (2) Nuclear-Chicago Mark 1:

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window setting Upper - 9.9, Lower - 0.5, and attenuator - c-550.

Ca^{2+} Analysis by Atomic Absorption

The concentration of $CaCl_2$ solution, which was added to the reaction mixture and the amount of endogenous Ca^{2+} present in preparations of the sarcoplasmic reticulum was analyzed by atomic absorption spectroscopy as described by Duggan and Martonosi (1970). A Perkin Elmer atomic absorption spectrometer, model 303 (Perkin Elmer, Norwalk, Connecticut) was used for Ca^{2+} measurement. A standard calcium solution was obtained from Fisher Scientific Company (Chicago, Illinois) and was used for calibration of the instrument in the presence of 10% trichloroacetic acid and 1% $LaCl_3$. The calcium solution was diluted to give 2 to 10 ppm of Ca^{2+} in 10% trichloroacetic acid and 1 % $LaCl_3$.

Determination of the amount of endogenous Ca^{2+} in the sarcoplasmic reticulum vesicles was performed by removing protein from the sarcoplasmic reticulum preparation in 10% trichloroacetic acid and 1% $LaCl_3$. The supernatant was used for Ca^{2+} analysis.

Protein Determination

The protein concentration was determined using the method of Lowry *et al.* (1951). Lowry solution A (20 g

Na_2CO_3, 12 g NaOH, 0.2 g $KNaC_4H_4O_6 \cdot 4H_2O$/l) was mixed at a ratio of 50 to 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 dilution (1:1) of Folin and Ciocalteu phenol reagent (Harleco, Philadelphia, Pennsylvania) 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 min at room temperature. A total of 0.5 ml of the diluted phenol solution was then added rapidly and mixed. It was allowed to stand with occasional shaking at room temperature for 45 min for color development. Absorbancy was measured at 660 nm against a control consisting of water plus all other reagents. The protein concentration was determined by comparing with a standard curve prepared from crystalline bovine serum albumin (Appendix Figure 1).

Enzyme Assays

The purity of the sarcoplasmic reticulum preparation was determined by measuring the activity of succinic-cytochrome c reductase, 5'-nucleotidase and acid phosphatase.

Succinic-cytochrome c reductase activity.

This enzyme was used as a marker enzyme to detect the presence of the mitochondrial membrane (Tisdale, 1967). The reaction mixture contained 100 µl of 0.1 M potassium

phosphate buffer (pH 7.4), 10 µl of 0.1 M NaN_3, 20 µl of 10 mM disodium ethylenediaminetetraacetate (EDTA), 50 µl of 10% bovine serum albumin, 100 µl of potassium succinate (pH 7.0) and 720 µl of distilled water.

The preparation of sarcoplasmic reticulum was diluted to a concentration of 100 - 200 µg protein/ml in a solution of 0.88 M sucrose and 5 mM potassium succinate. An aliquot of the sample preparation was incubated with the assay mixture for 2 min at 38°C. The reaction was initiated by addition of 100 µl of 1% ferricytochrome c, and the change in absorbancy was measured at 550 nm against a control lacking only the enzyme. Specific activity of the enzyme was calculated as follows:

$$\frac{\text{O.D./min/mg protein}}{18.5} = \text{µmoles cytochrome c reduced/min/mg protein}$$

where, $18.5 \times 10^{-6}\ M^{-1}\ cm^{-1}$ is the extinction coefficient for cytochrome.

5'-nucleotidase activity.

Contamination of the sarcolemma was investigated by measuring 5'-nucleotidase activity. The activity of the enzyme was assayed using the method of Michell et al. (1965). The preparation of the sarcoplasmic reticulum (less than 1 mg protein) was incubated for 15 min at 37°C in 2 ml of an assay mixture containing 100 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), 5 mM ATP and 10 mM sodium potassium tartrate. The reaction was stopped with 1 ml of 25% (w/v)

trichloroacetic acid. P_i (inorganic phosphate) was assayed in 1 ml of the supernatant by the method of Fiske and SubbaRow (1925).

Inorganic phosphate determination (Fiske and SubbaRow method).

One ml of 5 N sulfuric acid and 1 ml of 2.5% ammonium molybdate were added to 1 ml of the sarcoplasmic reticulum supernatant. One ml of reducing solution, which was made by dissolving 0.25 g of the powdered reagent (0.2 g of 1-amino-2-naphthol-4-sulfonic acid, 1.2 g of sodium bisulfite and 1.2 g of sodium sulfite in 100 ml of water) was then added. The volume was made up to 10 ml. After mixing again, the absorbancy was measured at 660 nm after standing 10 min. The Pi concentration was determined by comparing with a standard curve prepared from analytically pure KH_2PO_4 (Appendix Figure 2).

Acid phosphatase activity.

This enzyme was used as a marker enzyme to detect the presence of the lysosomes. The enzyme activity was assayed using an analytical and diagnostic kit for phosphatase (acid, alkaline and prostatic - Sigma Chemical Company, St. Louis, Missouri). A total of 0.5 ml of the acid (citrate) buffer (pH 4.8) was added to the Sigma phosphatase substrate (p-nitrophenyl phosphate). Then 0.2 ml of appropriately diluted sarcoplasmic reticulum suspension was added to the substrate mixture. After 30 min incubation at room temperature, 5 ml of 0.1 N NaOH was added and thoroughly

mixed to stop the reaction. The absorbancy of the mixture was read at 410 nm against the control lacking only the sarcoplasmic reticulum preparation. Units of acid phosphatase were determined from the standard curve (Appendix Figure 3). The unit value was converted to μmoles Pi/min/mg of protein.

SDS Gel Electrophoresis of Beef and Rabbit Sarcoplasmic Reticulum

Electrophoresis was performed using glass tubes (10.5 cm X 5 mm ID) in a Polyanalyst analytical polyacrylamide verticle disc gel electrophoresis apparatus (Buchler Instruments, #3-1750, Fort Lee, New Jersey). Voltage was regulated with a Heath kit High Voltage Power Supply, Model IP-17 (Heath Company, Benton Harbor, Michigan).

The sarcoplasmic reticulum of beef and rabbit was analyzed using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) by a modification of the method of Paterson and Strohman (1970).

Sample preparation.

Sarcoplasmic reticulum preparation was diluted to a protein concentration of 1 mg/ml in a solution containing 0.3 M sucrose, 0.6 M KCl and 10 mM histidine (pH 7.3), and kept on ice for 1 hour to remove contaminating myofibrillar protein. The vesicles were removed by sedimentation at 35,000 rpm for 1 hour in the SB-283 rotor in the IEC preparative ultracentrifuge. The pellet was

resuspended in 0.3 M sucrose with 2.5 mM HEPES buffer (pH 7.4). Solid SDS was added to the salt extracted sarcoplasmic reticulum at 1% of the total volume and dissolved by shaking. The protein solution was then dialyzed overnight at room temperature against 0.225 M Tris-glycine buffer (pH 8.6), containing 1% SDS. After dialysis, a small amount of glycerol and tracking dye (pyronin Y, 40 µg/ml of water) were added to the protein solution.

Gel preparation.

The gels (7.5% acrylamide with 0.15% Bis-acrylamide) were prepared using 10 ml of gel buffer (15.0 g Tris base, 72.0 g glycine/l), 7.5 ml of acrylamide solution (25.0 g acrylamide, 0.5 g Bis-acrylamide/100 ml), 2.5 ml of 50% glycerol, 1.0 ml of 1% TEMED (N,N,N',N'-tetramethylethylenediamine), 1.0 ml of 2.5% SDS solution, 1.0 ml of freshly prepared 1% ammonium persulfate and 2.0 ml of deionized water. All solutions were deairated prior to use using an aspirator. After mixing, the solution was poured into the glass tubes to a height of 8.5 - 9.5 cm. A solution consisting of an equivalent concentration of the buffer, TEMED and SDS, and a reduced concentration of ammonium persulfate (0.004%) was layered on top of the gel solution without disturbing the solution. The gel solution was then allowed to polymerize at room temperature for 20 - 30 min.

Sample application.

The sample (10 - 100 µl) was placed on top of the gel surface using a Lancer precision pipette (Sherwood Medical Industries, Inc., Bridgeton, Missouri).

Electrophoretic conditions.

The upper and lower chambers of the apparatus were filled with Tris-glycine buffer (pH 8.6, 3.0 g Tris base, 14.4 g glycine/l) having 0.1% SDS. Electrophoresis was performed at 20 volts per 12 tubes at room temperature for 16-17 hours or until the dye band migrated 75% of the gel length. The gels were removed from the tubes, and the front of the dye band was marked with a fine wire.

Fixing, staining and destaining.

The gels were fixed in the solution containing 3.5% perchloric acid and 10% isopropanol and let stand for 6 hours at room temperature.

The protein bands were determined by staining the gels in a solution of 0.02% coomassie brilliant blue R-250 in 50% methanol - 10% glacial acetic acid. After staining overnight, the background was diffused out in 5% methanol-5% glacial acetic acid using a BioRad model 170 gel electrophoresis diffusion destainer (BioRad Laboratories, Richmond, California).

After destaining, the relative mobility (Rm) was determined by dividing the protein migration distance by the distance that the marker dye migrated.

A standard curve for molecular weight determination was prepared using the following proteins: myoglobin (17,800), chymotrypsin (26,000), glyceraldehyde phosphate dehydrogenase (36,000), ovalbumin (43,000), catalase (60,000), bovine serum albumin (68,000), urease (83,000), phosphorylase a (94,000), C-protein (130,000), and myosin (220,000). The number indicates molecular weights of the reduced polypeptide chain.

Electron Microscopy

Sarcoplasmic reticulum vesicles from fresh and cold shortened sternomandibularis muscle were prepared for electron microscopic examination. Small pellets of the sarcoplasmic reticulum vesicles obtained by centrifugation were fixed for 2 hours with 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0). The pellets were then washed for 30 min in several changes of 0.1 M phosphate buffer (pH 7.0), and were fixed for 1 hour by the addition of 0.5 ml of 2% aqueous osmium tetraoxide.

After fixation, the samples were rinsed with the buffer and dehydrated for 20 min each in 30, 50, 70 and 95% ethanol. They were then placed in 2 changes of 100% ethanol for 20 min each. The dehydrated samples were transferred to propylene oxide for 30 min followed by 12 hours in a 1:1 mixture of propylene oxide and epon. The samples were then embedded in pure epon using flat embedding molds (LKB Instruments, Inc.).

The embedded blocks were trimmed by hand with a razor blade. The samples were then sectioned with a glass knife to a thickness of 6 to 8 μm using an LKB ultramicrotome. Sections were picked up from the knife boat on uncoated 300-mesh copper grids. Staining of the tissue sections was accomplished by floating the grids for 30 min on a saturated solution of uranyl acetate, rinsing thoroughly with distilled water, and then staining for 5 min in a solution of lead citrate (Reynolds, 1963). The sections were washed with distilled water and then dried.

Negative staining was accomplished by coating 300-mesh grids with a parodion film covered by a thin layer of evaporated carbon. A drop of the suspended sarcoplasmic reticulum vesicles was applied to the grid followed by drying. The dried sample was then washed with distilled water to remove sucrose contained in the sample solution. The sample was negatively stained using 2% phosphotungstic acid. The last drop of phosphotungstic acid was removed by touching the grid edge to a filter paper followed by drying.

A Philips EM-300 electron microscope was used for observing the stained sections and negatively stained materials at an accelerating voltage of 60 KV. Representative photographs of each sample were taken on Kodak Ester Thick base-70 mm film. The film was developed for 3 min in a Kodak D-19 developer, washed for 30 sec in running water, fixed in a Kodak fixer for 4 min, washed for 30 min in running water, washed in distilled water for 2 min and then dried.

The negatives were printed on Ilford Ilfoprint rapid stabilization paper using a Durst S-45-EM enlarger. The prints were then developed in an Ilford model 1501 rapid stabilization processor. Selected prints were fixed in a Kodak fixer, washed and dried on a ferrotype dryer.

RESULTS AND DISCUSSION

Isolation of Sarcoplasmic Reticulum Vesicles

Separation of sarcoplasmic reticulum vesicles on sucrose gradient.

The density gradient profiles of sarcoplasmic reticulum vesicles prepared from fresh and cold shortened muscles are shown in Figure 3. The fresh muscle preparations usually had a broad continuous band in the upper half of the gradient and a narrower band at the interface between 33.9 and 37.2% sucrose solution. The only difference noted between fresh and cold shortened muscle preparations was the marked reduction in the size of the lower band.

The density gradient profile of sarcoplasmic reticulum vesicles prepared from muscle stored for 24 hours at 15°C had the same pattern as that of cold shortened muscle. This indicated that the difference in profiles was not due to cold shortening *per se*, since non-cold shortened muscle also gave the same profile.

Yield of sarcoplasmic reticulum vesicles.

Two hundred grams of ground muscle were used for each preparation. The yield of sarcoplasmic reticulum vesicles

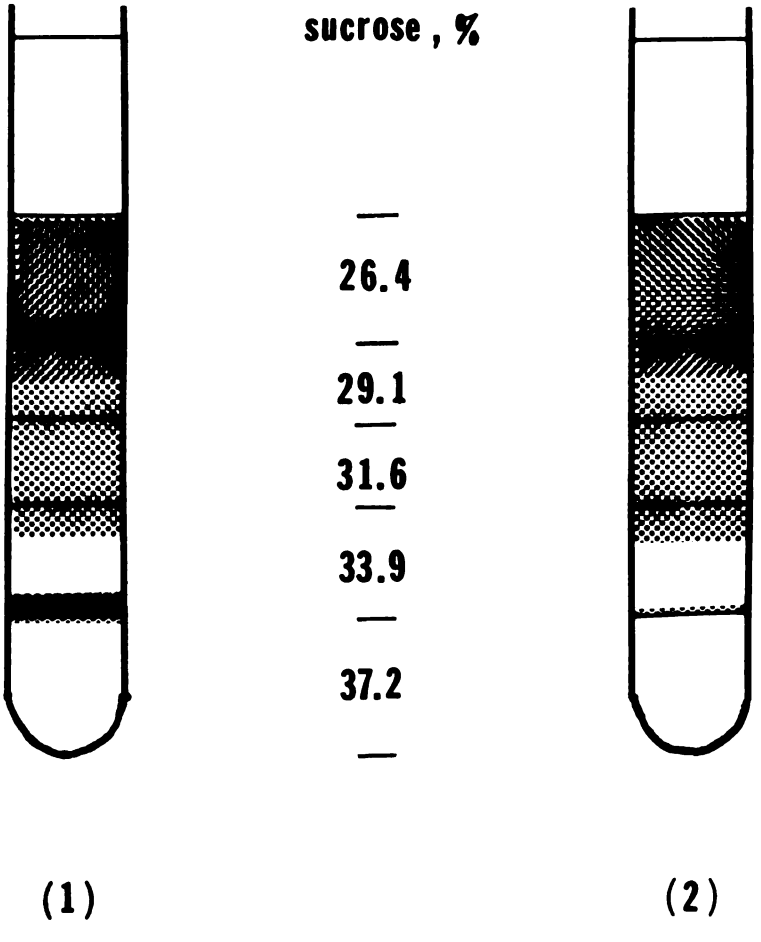


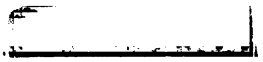
Figure 3. Density gradient profiles of the sarcoplasmic reticulum from fresh and cold shortened muscle ([1] fresh muscle preparation; [2] cold shortened muscle preparation).

from fresh beef muscle was 42.3 ± 8 μg per gram of ground muscle, whereas, that from rabbit longissimus dorsi muscle was 380 ± 40 μg per gram of ground muscle. Thus, the yield of rabbit sarcoplasmic reticulum vesicles was about 10 fold greater than that of beef. The large difference in yield may be caused by a greater amount of connective tissue in beef sternomandibularis muscle, which may interfere with isolation of the sarcoplasmic reticulum.

The yield from cold shortened beef muscle was even lower (25 ± 4 μg/g muscle) than fresh muscle. However, the yield from the muscle stored for 24 hours at 15°C was still lower (about 16 μg/g muscle) than that from cold shortened muscle. Therefore, it is postulated that the decreased yield from post-mortem muscle is not due to difficulty in isolation of the sarcoplasmic reticulum, but rather to the action of enzymes on the sarcoplasmic reticulum during post-mortem storage. The higher value for cold shortened muscle (0°C) as compared to that held at 15°C for 24 hours may be the result of a greater amount of proteolysis at the higher temperature.

Purity of sarcoplasmic reticulum preparation.

Succinate cytochrome c reductase activity was used for monitoring the contamination from mitochondrial membranes. The specific activity was 0.007 μmoles of cytochrome c reduced per min per mg protein. Assuming a reducing rate for purified mitochondria of 0.5 μmoles/min/mg protein (Meissner and Fleischer, 1971), the specific activity obtained would indicate only 1.4%



contamination by mitochondria.

Acid phosphatase activity of sarcoplasmic reticulum preparations was 0.015 μmoles Pi per min per mg protein. Meissner and Fleischer (1971) determined an activity of 0.002 to 0.005 μmoles per min per mg protein in rabbit sarcoplasmic reticulum. Based on these values, the preparations utilized in the present study were more contaminated with lysosomes than their preparations. They stated that the range of the acid phosphatase activity indicated negligible contamination by lysosomes in their study. Although the values in the present study were slightly higher, only slight contamination was indicated.

The amount of 5'-nucleotidase activity was negligible for the preparations utilized in this investigation.

Results indicated that the sarcoplasmic reticulum prepared in this study was only slightly contaminated by mitochondria and lysosomes, and showed negligible contamination from the sarcolemma. However, the sarcoplasmic reticulum preparation may be contaminated by myofibrillar protein, since salt extraction of the preparation by a solution containing 0.6 M KCl, 0.3 M sucrose and 10 mM histidine (pH 7.3) was not performed. Although attempts were made to extract the sarcoplasmic reticulum preparation with this solution to remove the myofibrillar proteins, it caused inactivation of the Ca^{2+} accumulating ability and could not be used.

Ca^{2+} Accumulation as a Function of Sarcoplasmic Reticulum Protein Concentration

Figure 4 shows the relationship between the amount of accumulated Ca^{2+} and protein concentration. A linear relationship was found between 16 and 80 µg protein per ml of reaction mixture, but the linear curve did not extrapolate to zero. However, no correction was made for the amount of accumulated Ca^{2+} per mg of sarcoplasmic reticulum protein on this graph. Because the amount of accumulated Ca^{2+} was approximately proportional to the protein concentration between 40 and 80 µg, the measurement of Ca^{2+} accumulation and release was taken within this range.

Stability of Isolated Sarcoplasmic Reticulum Vesicles

Sarcoplasmic reticulum vesicles obtained from muscle immediately after death have been shown to lose their activity during storage at 0°C and neutral pH (7.0 to 7.4), with losses from 0 to 50% being reported during the first day after isolation (Ebashi and Lipmann, 1962; Muscatello et al., 1962; Lee et al., 1965; Eletr and Inesi, 1972). In this study, isolated sarcoplasmic reticulum vesicles were fairly stable in activity for 90 min storage in 0.3 M sucrose and 2.5 mM HEPES buffer (pH 7.4) at 0°C (Figure 5).

Results of stability tests on the isolated sarcoplasmic reticulum preparation is shown in Figure 6. Even at

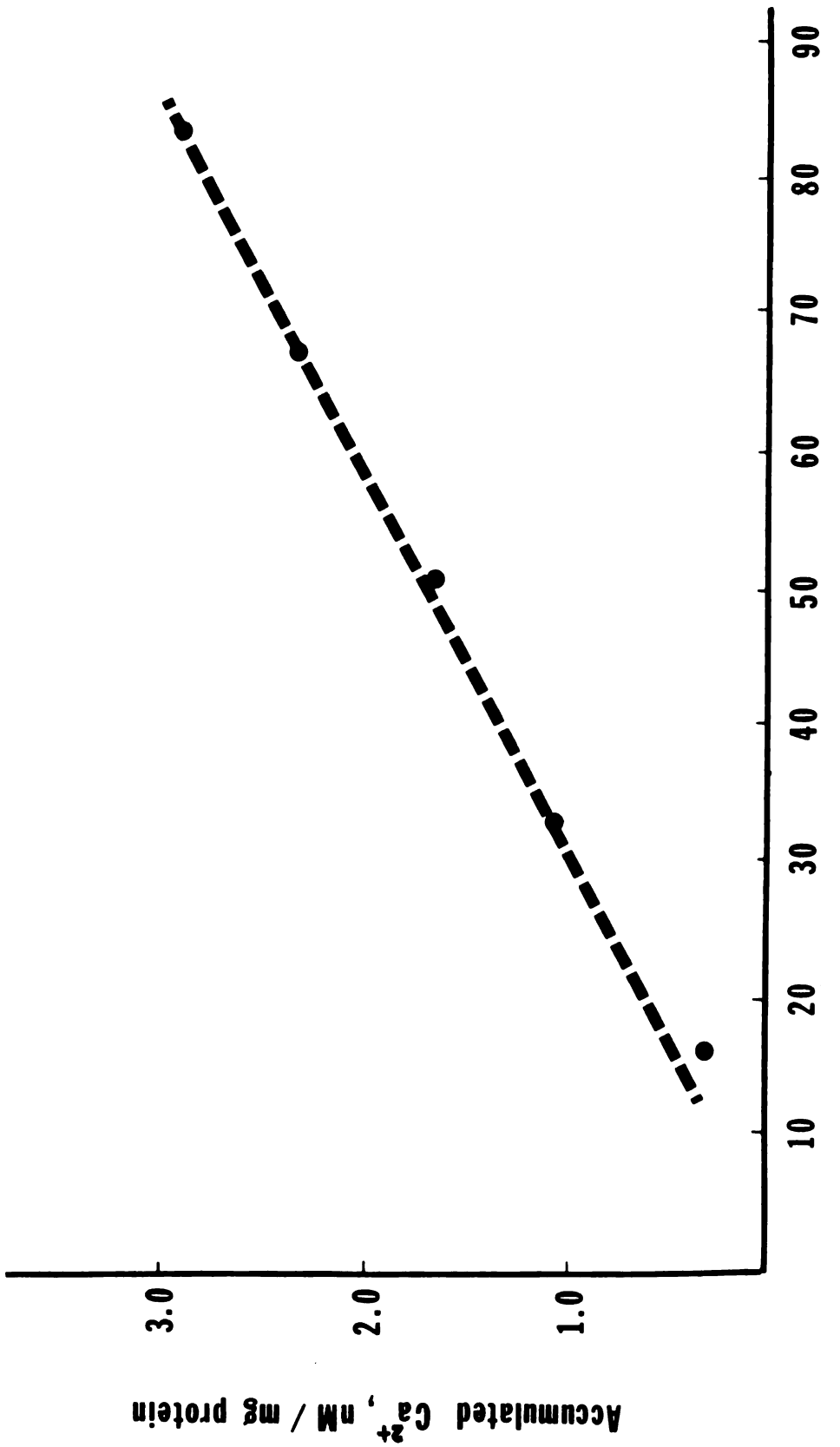


Figure 4. Ca^{2+} accumulation of sarcoplasmic reticulum vesicles as a function of protein concentration carried out in HEPES buffer (pH 7.3) at 38°C.

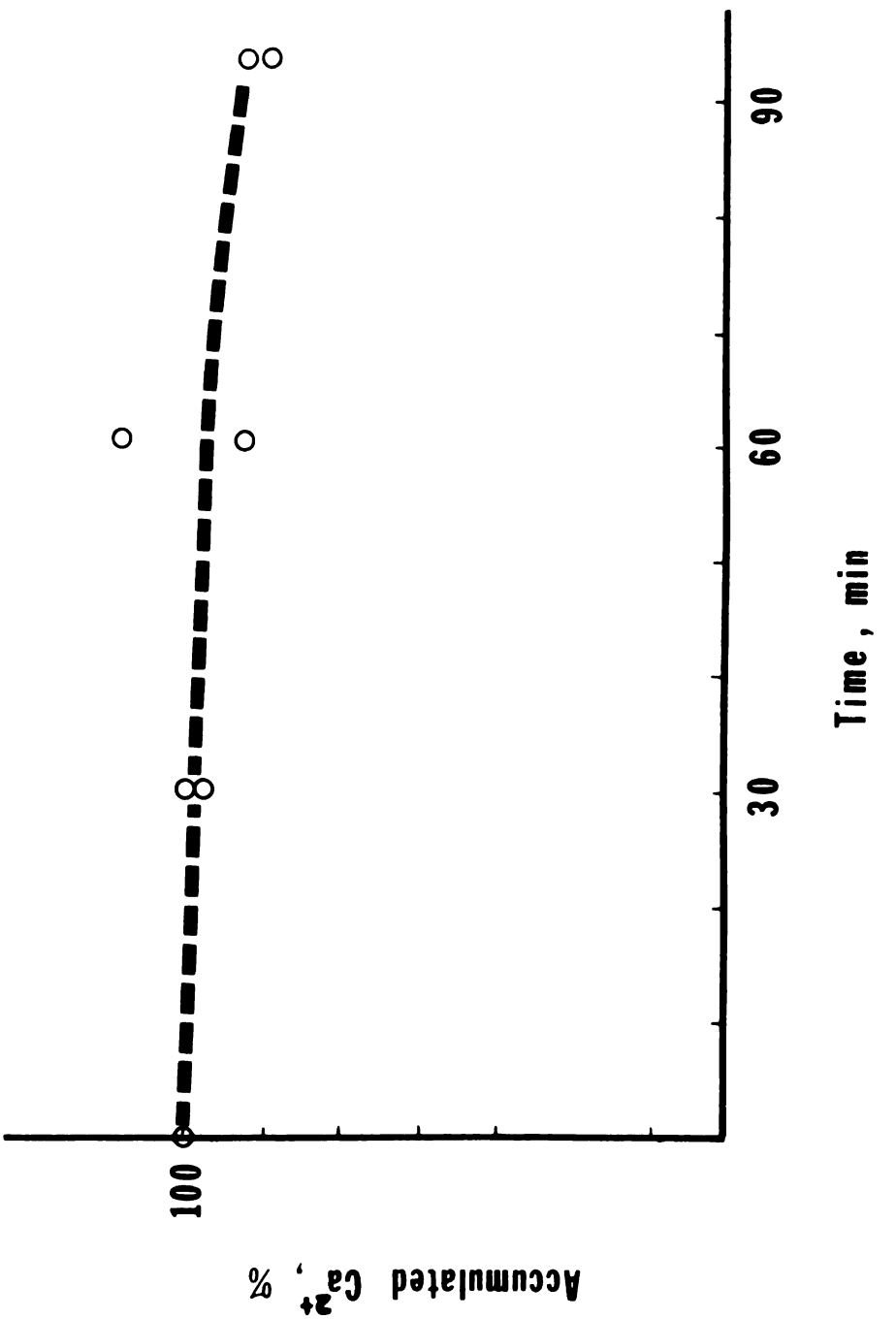


Figure 5. Stability of sarcoplasmic reticulum vesicles carried out in HEPES buffer (pH 7.3) at 0°C.

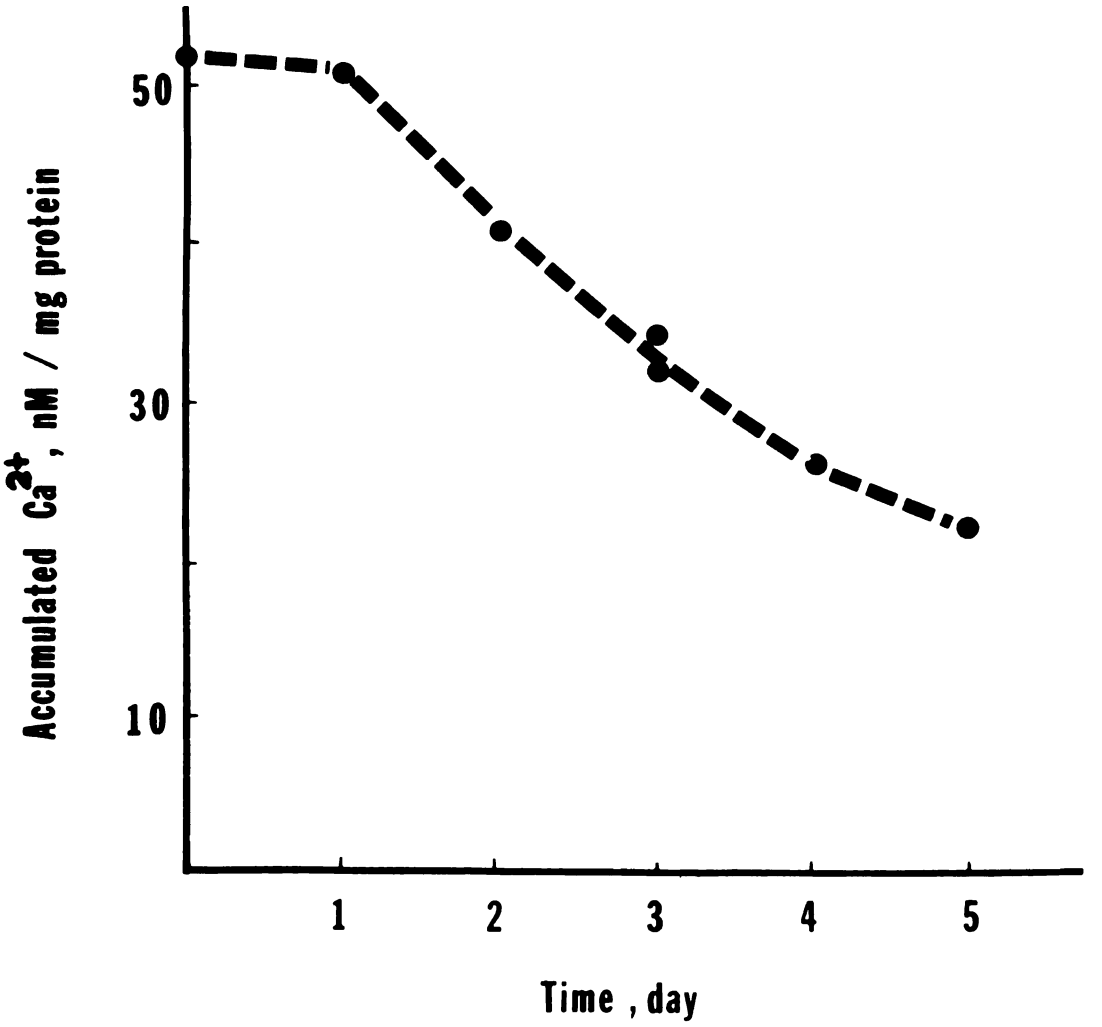


Figure 6. Stability of sarcoplasmic reticulum vesicles at -20°C.

-20°C storage, the sarcoplasmic reticulum vesicles lost their activity rather rapidly. Since the storage temperature used during this test fluctuated in the range from -12 to -20°C, ice crystals could grow and impart conformational changes to the proteins, causing inactivation of the Ca^{2+} accumulating proteins.

From the results of two stability tests and information about the stability of isolated sarcoplasmic reticulum in the literature (Ebashi and Lipmann, 1962; Muscatello et al., 1962; Lee et al., 1965; Eletr and Inesi, 1972), determination of Ca^{2+} accumulation and Ca^{2+} release was performed immediately after isolation of the sarcoplasmic reticulum vesicles.

Endogenous Ca^{2+} Concentration

The amount of endogenous Ca^{2+} bound by the sarcoplasmic reticulum was found to be 16 nM/mg protein. This value is low compared to 35 $\pm$ 5 nM Ca^{2+}/mg protein for rabbit sarcoplasmic reticulum as reported by Meissner et al. (1973). Chevallier and Butow (1971) have observed that the endogenous Ca^{2+} concentration in rabbit sarcoplasmic reticulum is 500 nM/mg protein. The differences reported in endogenous Ca^{2+} concentration may be caused by variations in the isolation procedures and differences between species.

Ca^{2+} Accumulation

Ca^{2+} accumulation as a function of reaction time and temperature.

Figure 7 shows Ca^{2+} accumulation of the sarcoplasmic reticulum as a function of reaction time. This graph indicates that Ca^{2+} accumulation begins immediately upon addition of the sarcoplasmic reticulum and is completed within 1 minute. At 0 and 15°C, the amount of accumulated Ca^{2+} was gradually released with the passage of time. After 20 min reaction time at 15°C, approximately 8% of the accumulated Ca^{2+} was released from the sarcoplasmic reticulum vesicles. About 15% of the accumulated Ca^{2+} was released by holding the sarcoplasmic reticulum vesicles for 30 min at 0°C. A similar phenomenon in Ca^{2+} release from saturated sarcoplasmic reticulum was also observed by Harigaya and Schwaltz (1974).

On the other hand, the sarcoplasmic reticulum vesicles were unstable at 38°C (Figure 7). Approximately 50% of the accumulated Ca^{2+} was released during a 10 min reaction time. Johnson and Inesi (1969), Inesi et al. (1973) and Sreter (1969) reported that Ca^{2+} release from Ca^{2+} saturated vesicles occurred more rapidly after mild heat denaturation (30 - 50°C) of fragmented sarcoplasmic reticulum. If the temperature was increased above 35°C, the amount of accumulated Ca^{2+} dropped sharply (Inesi et al., 1973). This is in contrast to the work of Greaser et al. (1969), who observed that raising the temperature to 37°C at pH 7.2 before

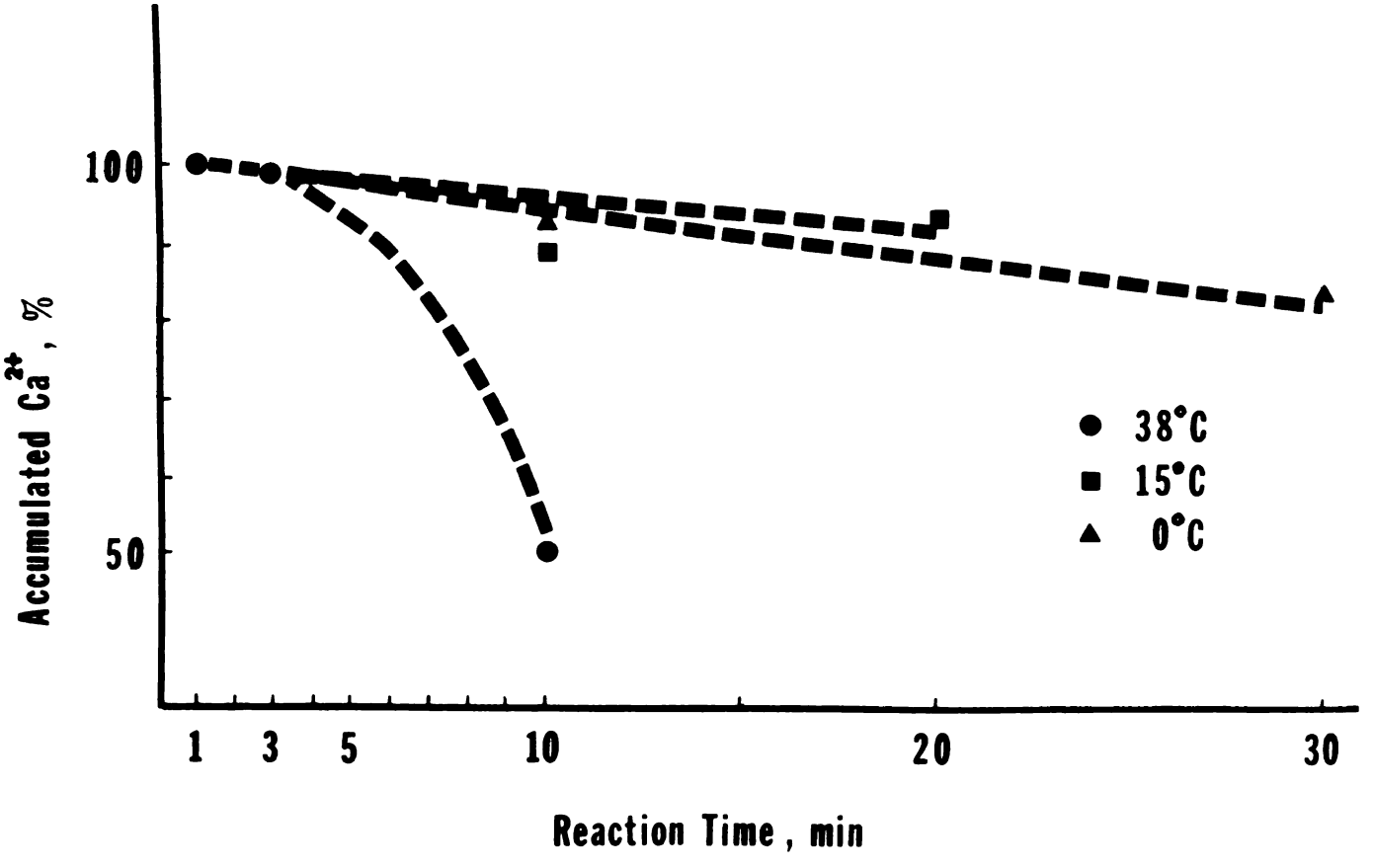


Figure 7. Ca^{2+} accumulation of sarcoplasmic reticulum vesicles as a function of reaction time (pH 7.3).

the Ca^{2+} accumulation assay was relatively ineffective in reducing calcium accumulation in sarcoplasmic reticulum fragments. Results indicate that the sarcoplasmic reticulum vesicles are extremely unstable at 30 - 50°C, if they are saturated with Ca^{2+} ions. These observations may explain the shortening phenomenon taking place in sternomandibularis muscle at temperatures above 30°C.

Ca^{2+} accumulation.

Sarcoplasmic reticulum vesicles were isolated from fresh muscle, cold shortened muscle, and muscle stored for 24 hours at 15°C. The Ca^{2+} accumulating ability of each sarcoplasmic reticulum preparation was determined at pH 7.3 and 38°C for 3 min in the reaction mixture, and the results are shown in Table 1. Fresh muscle sarcoplasmic reticulum vesicles had 50.7 $\pm$ 2.6 nM of Ca^{2+}/mg protein. Harigaya and Schwaltz (1974) observed that the Ca^{2+} accumulating ability of the sarcoplasmic reticulum from rabbit red muscle was 83 nM/mg protein in the absence of Ca^{2+} precipitating agents. Although this value is higher than that of beef sternomandibularis muscle, the values are not comparable, because the protein components separated by SDS gel electrophoresis from beef and rabbit sarcoplasmic reticulum seem to differ from each other except for the ATPase fraction.

The sarcoplasmic reticulum from cold shortened muscle (0°C for 24 hours) showed about 75% of the Ca^{2+} accumulating ability of fresh muscle sarcoplasmic reticulum, whereas, no activity was detected in the sarcoplasmic reticulum

Table 1. Ca^{2+} accumulation of sarcoplasmic reticulum vesicles from fresh and cold shortened muscle and muscle stored for 24 hours at 15°C.

S.R. Source	Accumulated Ca^{2+} (nM/mg protein)[a]
fresh muscle	50.7 ± 2.6
cold shortened muscle	39.1 ± 1.3
muscle stored 24 hrs at 15°C	0

[a] The Ca^{2+} determination was performed for 3 min at pH 7.3 and 38°C. Each value represents the average of four determinations from two different muscle preparations.

vesicles from muscle stored for 24 hours at 15°C. Greaser et al. (1967) also observed a marked decrease in the Ca^{2+} binding activity of the sarcoplasmic reticulum from pig muscle with increasing post-mortem time. They reported that the Ca^{2+} accumulating activity had fallen to very low levels (less than 5% of the initial activity) by 24 hours post-mortem at 4°C.

Goll et al. (1971) also reported that fragmented sarcoplasmic reticulum prepared from rabbit psoas major muscle strips at the beginning of isometric tension possessed only 20 to 25% of the Ca^{2+} accumulating ability of the fresh (at-death) sarcoplasmic reticulum fraction. Furthermore, they found that short tryptic digestion caused a marked loss in the Ca^{2+} accumulating ability of fragmented sarcoplasmic reticulum, and at the same time, resulted in a slight increase in the Ca^{2+} stimulated, Mg^{2+} dependent ATPase activity of the preparations. From these results, they suggested that the main cause of loss in the Ca^{2+} accumulating ability of the sarcoplasmic reticulum after death is due to the uncoupling of the Ca^{2+} pump by proteolysis.

Effect of pH and temperature upon Ca^{2+} accumulation.

In living muscle tissue, ATP is mostly produced by aerobic oxidation of glucose (Bate-Smith, 1948). After death, oxygen is no longer supplied by the circulatory system, but the muscle tissue produced ATP by means of anaerobic glycolysis in which one molecule of glucose is

converted into 2 molecules of lactic acid and ATP. This reaction occurs in normal living muscle in times of stress. In living tissue, the lactic acid produced is carried away by the blood stream and metabolized. After death, however, lactic acid accumulates, resulting in a lowering of the pH of the sarcoplasm. The conversion of glycogen to lactic acid continues until a pH is reached at which the glycolytic enzymes are inactivated.

The pH changes in beef sternomandibularis muscle stored at 0°C are shown in Figure 8. After 24 hours storage, the pH of the muscle had declined to 5.8. During the first 3 hours post-mortem, the pH dropped rapidly to 6.4. This rapid drop may play an important role in cold shortening. Locker and Hagyard (1963) have stated that shortening occurred almost immediately when muscle (sternomandibularis) was stored at 0°C. At high temperatures, however, there was a delay in shortening. This means that a rapid pH decline coincident with a rapid drop in body temperature results in shortening.

Figure 9 shows Ca^{2+} accumulation at different pHs and temperatures. The Ca^{2+} accumulating ability decreased with decreasing pH values at all temperatures (0, 15 and 38°C). Sarcoplasmic reticulum vesicles accumulated 50 nM of Ca^{2+} per mg protein at pH 7.3 and 38°C, but accumulated only about one-fourth as much calcium at the same pH and 0°C. The Ca^{2+} accumulation at pH 7.3 and 15°C was approximately three-fourths of that at 38°C and pH 7.3. The

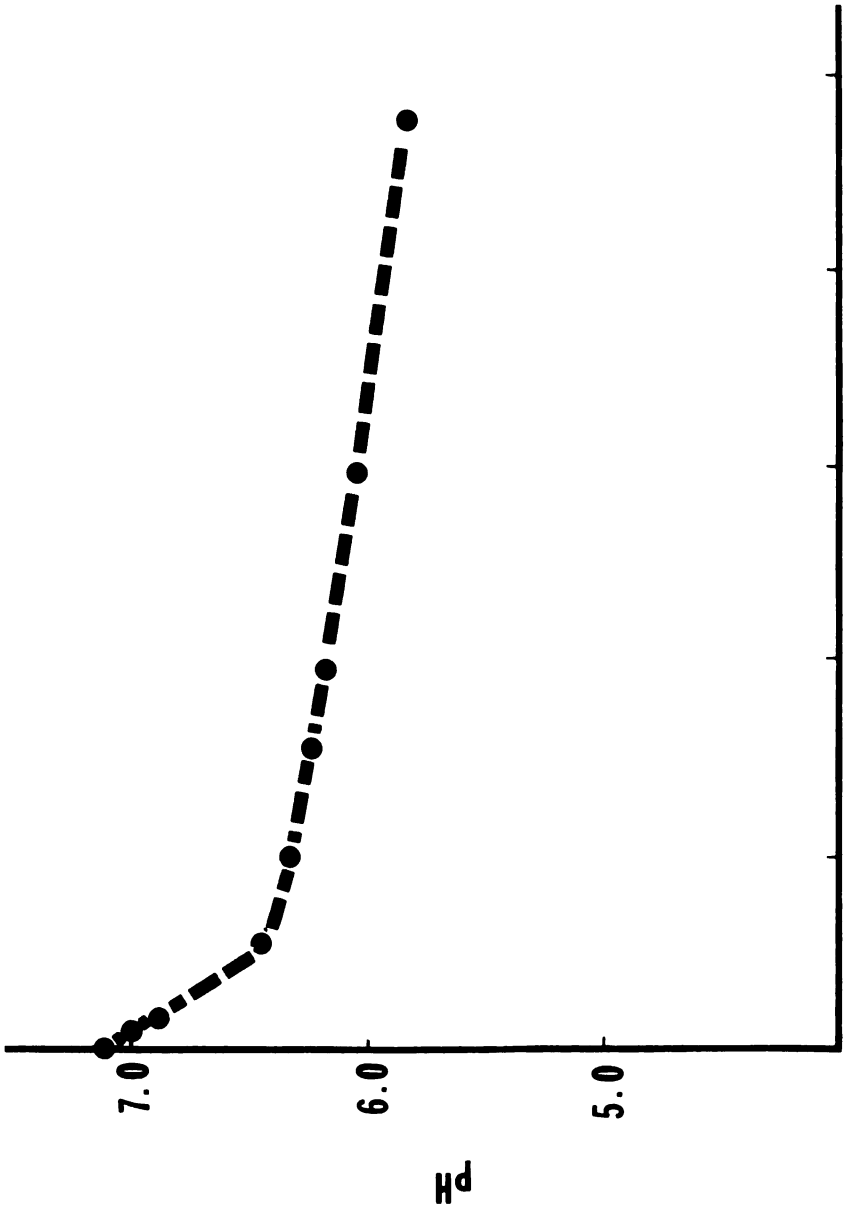


Figure 8. pH changes in beef sternomandibularis muscle at 0°C.

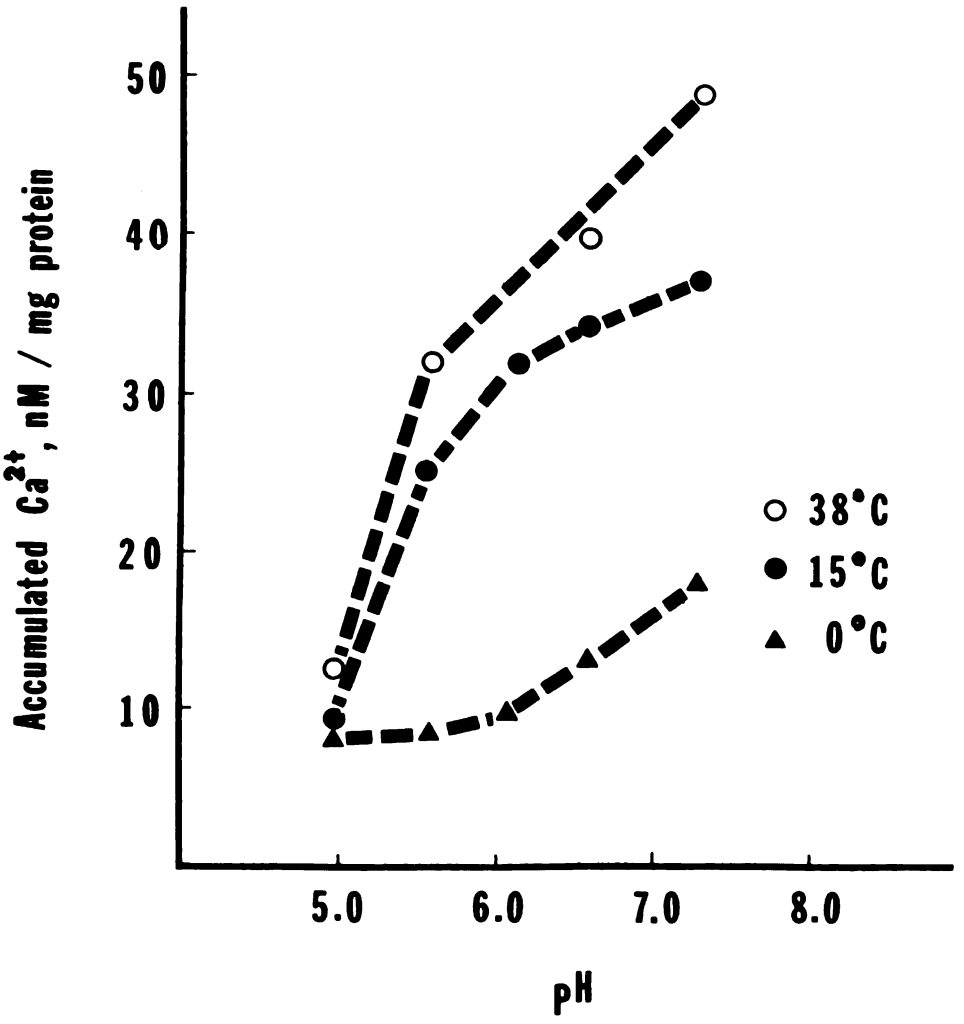


Figure 9. Ca^{2+} accumulation of sarcoplasmic reticulum vesicles at different pH values and temperatures.

Ca^{2+} accumulating ability at pH 5.0 was about 10 nM/mg protein regardless of temperature. This indicates that the Ca^{2+} accumulating ability of the sarcoplasmic reticulum is greatly inhibited at low pH values (5.0) regardless of temperature.

The maximum Ca^{2+} accumulating ability (50 nM of Ca^{2+}/ mg protein) in the pH range from 5.0 to 7.3 was obtained at the highest pH, which is equivalent to that in living muscle (Bate-Smith, 1948). Sreter (1969) stated that Ca^{2+} uptake by the sarcoplasmic reticulum from rabbit white muscle in the absence of oxalate was optimal between pH 5.6 - 6.5, reaching a maximum value of 240 - 250 nM Ca^{2+}/ mg protein. However, at pH 7.5 Ca^{2+} uptake was only about 40% of the maximum. This indicates that the sarcoplasmic reticulum from beef sternomandibularis muscle has different Ca^{2+} binding properties than that of rabbit white muscle. Figure 9 also shows that if the pH of the reaction mixture is lowered below 7.3, some of the accumulated Ca^{2+} is released from the vesicles.

Ca^{2+} Release

Ca^{2+} release from Ca^{2+} saturated sarcoplasmic reticulum was determined using fresh muscle sarcoplasmic reticulum preparations. The effects of temperature on Ca^{2+} release at different pH values is shown in Figure 10. The bar graph shows the amount of accumulated Ca^{2+} in nM/mg protein under different conditions.

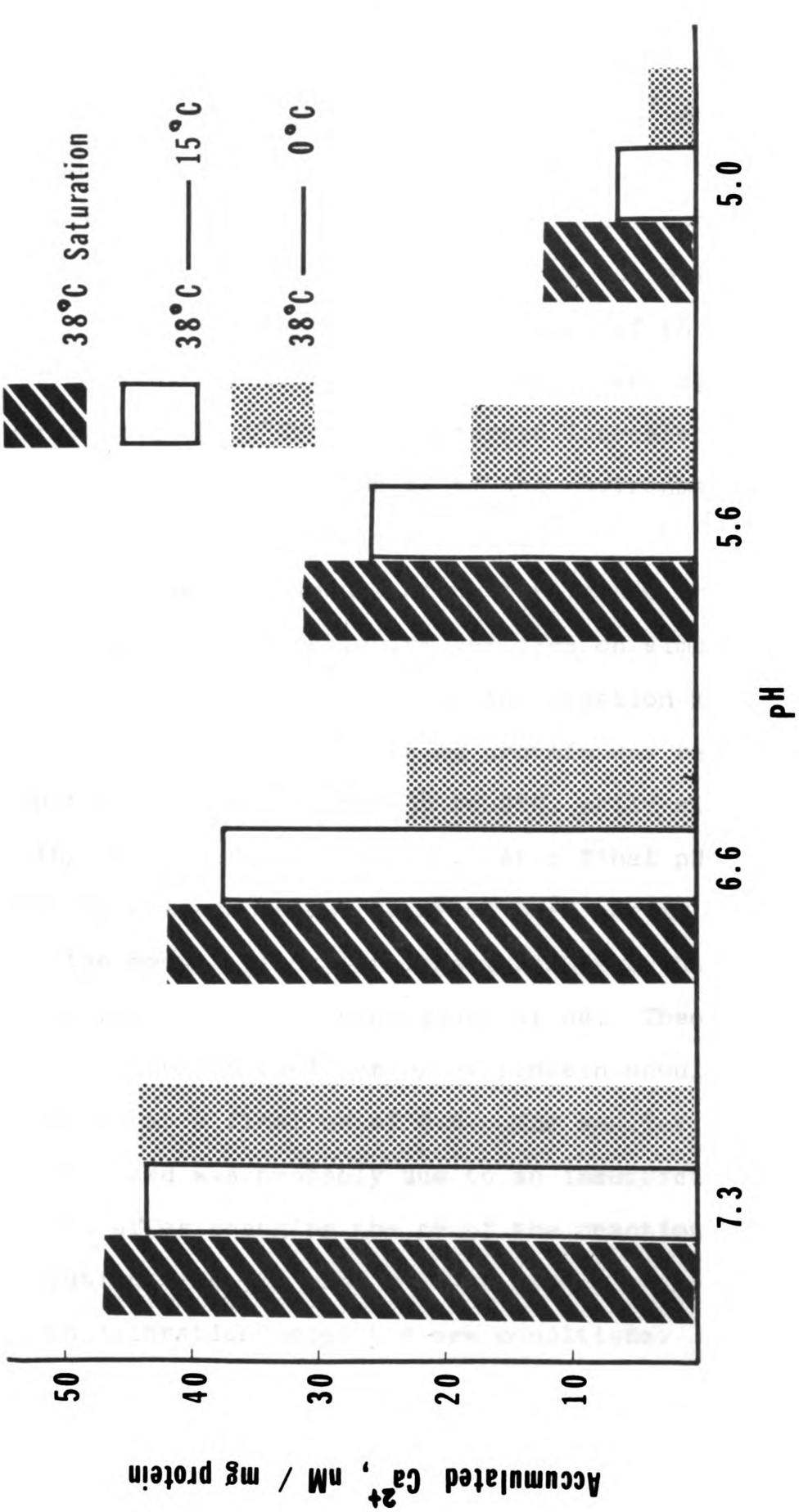


Figure 10. Effect of pH and temperature on Ca^{2+} release from saturated sarcoplasmic reticulum vesicles.

The bar graph (Figure 10) indicates that the temperature change did not affect the release of Ca^{2+} at physiological pH (7.3). On the other hand, about 48% of the bound Ca^{2+} was released at pH 6.6 by changing the temperature from 38 to 0°C. Lowering the temperature from 38 to 15°C at the same pH resulted in the release of 12% of the total bound Ca^{2+}. Thus, results indicate that lowering the temperature from 38 to 0°C caused a marked increase in the amount of Ca^{2+} release if the pH of the environment was lowered below the physiological value.

Table 2 shows the amount of Ca^{2+} released from Ca^{2+} saturated sarcoplasmic reticulum vesicles on simultaneously changing the pH and temperature of the reaction mixture. Approximately 10 nM of Ca^{2+} per mg protein was released on lowering the temperature from 38 to 0°C, while simultaneously decreasing the pH from 7.3 to 6.6. At a final pH of 5.0, the vesicles released about 30 nM of Ca^{2+} per mg of protein. However, the amount of Ca^{2+} released was less than expected in view of the effect of temperature alone. Theoretically about 20 or 25 nM of Ca^{2+} per mg of protein should have been released at a final pH of 6.6. The smaller amount of Ca^{2+} released was probably due to an insufficient incubation time after changing the pH of the reaction mixture. The incubation time (10 min) may not have been long enough to give equilibration under the new conditions. Greaser et al. (1969a) observed that it was necessary to hold the sarcoplasmic reticulum suspension for one hour after altering

Table 2. Ca^{2+} release from Ca^{2+} saturated sarcoplasmic reticulum vesicles* on simultaneously changing the pH and temperature of the reaction mixture.

Final pH	Temperature**	Released Ca^{2+} (nM/mg protein)***
6.6	38 ⟶ 0°C	41.4 ± 3.7
5.6	38 ⟶ 0°C	23.2 ± 0.1
5.0	38 ⟶ 0°C	29.3 ± 5.6

*Saturated sarcoplasmic reticulum vesicles had 50.7 ± 2.6 nM of Ca^{2+} per mg protein.

**Temperature was changed from 38 to 0°C.

***Each value represents the average of two determinations from one muscle preparation.

the pH in order to attain equilibration.

The incubation time (10 min) chosen for this study was selected after examining Figure 7, which shows the curve at 0°C gradually declined with increasing reaction times. After a 30 min reaction time, about 15% of the accumulated Ca^{2+} had been released, therefore, a 10 minute reaction time was adopted for this experiment.

Greaser et al. (1969a) investigated the effect of pH-temperature treatments on the calcium accumulating ability of porcine sarcoplasmic reticulum. After the pH-temperature treatment, the sarcoplasmic reticulum suspension was brought back to pH 7.2 with 0.1 N KOH. The Ca^{2+} accumulating ability was then assayed. They found that treatment at pH 4.5 and 0°C for 1 hour abolished the majority of calcium uptake, and that inactivation by pH was greater at higher temperatures. Thus, they concluded that the combined effect of temperature and pH may explain inactivation of the Ca^{2+} accumulating ability of the sarcoplasmic reticulum that they had observed earlier (Greaser et al., 1967, 1969b), and that the loss of activity may be related to the rate of ATP breakdown and development of rigor mortis in post-mortem muscle.

Although there are differences in species and experimental procedures between the present study and that of Greaser et al. (1969a), the results of the present study indicate that pH and temperature treatment altered the Ca^{2+} accumulating ability of the sarcoplasmic reticulum.

Lowering the pH and temperature simultaneously resulted in a decreased Ca^{2+} accumulating capacity in beef sarcoplasmic reticulum and appears to be equivalent to cold shortening.

SDS Gel Electrophoresis of Beef and Rabbit Sarcoplasmic Reticulum

SDS gel electrophoresis using 7.5% acrylamide gels was performed on salt-extracted and unextracted beef sarcoplasmic reticulum preparations. Rabbit sarcoplasmic reticulum was also examined by the SDS gel electrophoresis in order to compare the protein components with beef sarcoplasmic reticulum. Rabbit sarcoplasmic reticulum was isolated from fresh longissimus dorsi muscle and extracted by a solution containing 0.6 M KCl, 0.3 M sucrose and 10 mM histidine (pH 7.3) to remove the myofibrillar proteins.

Protein profile of beef sarcoplasmic reticulum.

Relative mobilities (Rm) on the SDS gel of salt-extracted and unextracted beef sarcoplasmic reticulum are shown in Table 3. The SDS gel pattern for salt-extracted beef sarcoplasmic reticulum consists of four major protein components having Rm values of 0.25, 0.40, 0.43 and 0.96. The component at Rm of 0.96 gives a broad diffuse band of an opalescent color. In addition, some minor components were observed in the Rm range from 0.55 to 0.64, but their diffuse nature made it difficult to give a good estimate of their precise positions. Unextracted beef sarcoplasmic reticulum

Table 3. Relative mobilities (Rm) of protein components from beef sarcoplasmic reticulum (salt-extracted and unextracted) and salt-extracted rabbit sarcoplasmic reticulum.

	Rm value(1
Salt-extracted beef S.R.(2	0.26, 0.40, 0.43, 0.59-0.65, 0.96(3
Unextracted beef S.R.(2	0.26, 0.30, 0.32, 0.41, 0.44, 0.58, 0.59-0.64, 0.97(3
Salt-extracted rabbit S.R.(2	0.27, 0.29, 0.31, 0.37 0.45, 0.55-0.66, 0.78, 0.97(3

(1 Value is the average of four measurements.

(2 S.R. represents the sarcoplasmic reticulum.

(3 Value represents the center of a broad diffuse band.

gave a few faint bands having Rm values of 0.30, 0.38 and 0.58 in addition to the protein bands described earlier. Thus, results indicate that the unextracted beef sarcoplasmic reticulum, which was used for determination of the Ca^{2+} accumulating ability, although slightly contaminated by myofibrillar protein, is relatively pure.

Molecular weight estimation.

The SDS gel electrophoresis patterns of beef and rabbit sarcoplasmic reticulum are shown in Figure 11. The molecular weights of the various protein components of both beef and rabbit sarcoplasmic reticulum were estimated from the standard curve for molecular weight estimation (Figure 12).

1) Beef sarcoplasmic reticulum: Major components of beef sarcoplasmic reticulum occurred at Rm values of 0.25, 0.40, 0.43 and 0.96. The first three components correspond to molecular weights of 100,000, 63,000 and 58,000 daltons on SDS gels. The diffuse nature of the protein at Rm 0.96 made it impossible to give a good estimation of its molecular weight, although it was less than 10,000.

2) Rabbit sarcoplasmic reticulum: The SDS gel pattern for rabbit sarcoplasmic reticulum gave four major bands (Figure 11). Their molecular weights were estimated to be 100,000, 68,000, 54,000 and less than 10,000. Although the protein components from beef sarcoplasmic reticulum have not been positively identified, there is considerable information available on the protein components of rabbit sarcoplasmic reticulum. The 100,000 molecular weight protein

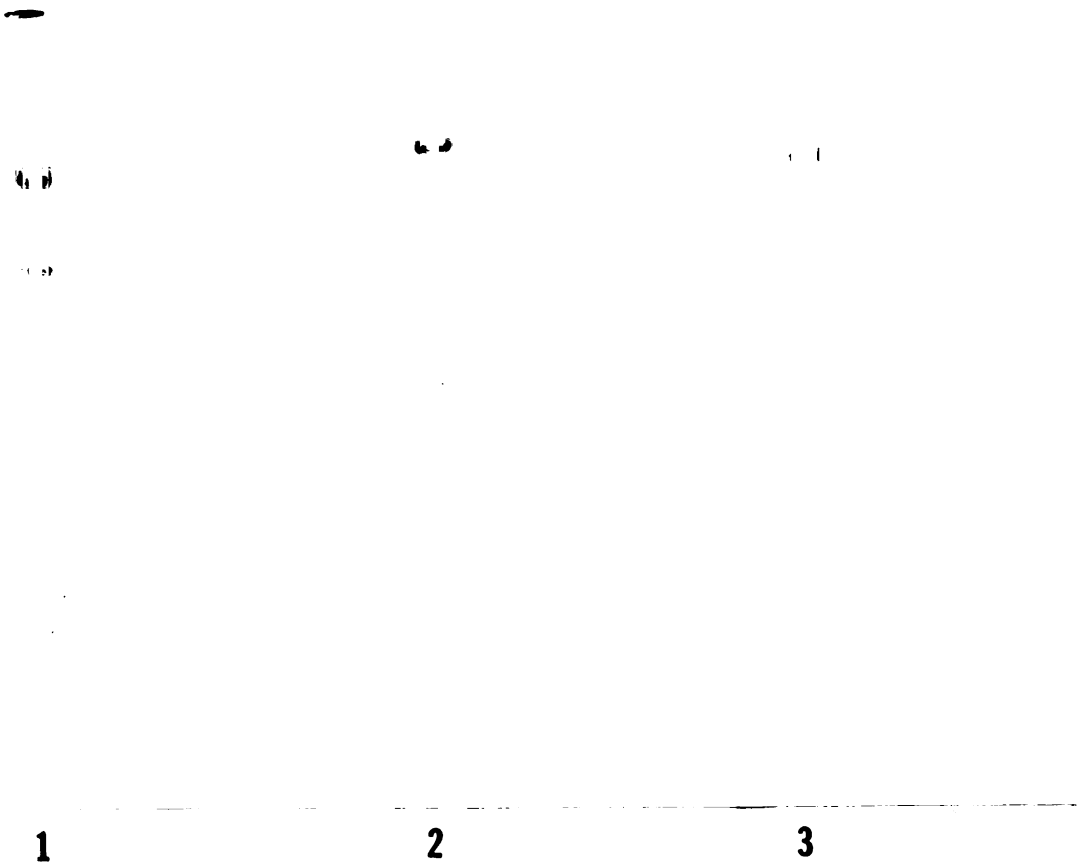


Figure 11. The SDS gel electrophoresis patterns of beef (salt-extracted and unextracted) and salt-extracted rabbit sarcoplasmic reticulum ([1] unextracted beef S.R.; [2] extracted beef S.R.; [3] extracted rabbit S.R.).

was the major component and is presumed to be Ca^{2+} activated ATPase (Martonosi, 1968; Salinger and Klein, 1969; MacLennan, 1970; Inesi et al., 1970; McFarland and Inesi, 1970; Martonosi and Halpin, 1971; MacLennan and Seeman, 1971; Meissner and Fleisher, 1971).

The 54,000 molecular weight protein is probably Calsequestrin. Although Calsequestrin has been reported to have a molecular weight of 46,500 by MacLennan and Wong (1971), Ostwald and MacLennan (1974) have shown that it is identical with the 55,000-dalton protein reported by Ikemoto et al. (1972). The discrepancy in the molecular weight of this protein is probably due to the use of different molecular weight standards. The 68,000 molecular weight protein is probably identical to the high affinity Ca^{2+} binding protein identified by MacLennan et al. (1972).

The protein band of rabbit sarcoplasmic reticulum observed near the front of the marker dye is likely proteolipid (MacLennan et al., 1972). In addition to those four bands, a few other faint bands were observed in the gel pattern. A broad band at Rm 0.55 - 0.66 may correspond to the Ca^{2+} binding acidic proteins identified by MacLennan et al. (1972).

3) Comparison of beef and rabbit sarcoplasmic reticulum: In both beef and rabbit sarcoplasmic reticulum, the 100,000 molecular weight protein was found, and is presumably the Ca^{2+} activated ATPase. The protein components having molecular weights of 68,000 and 54,000, which are observed

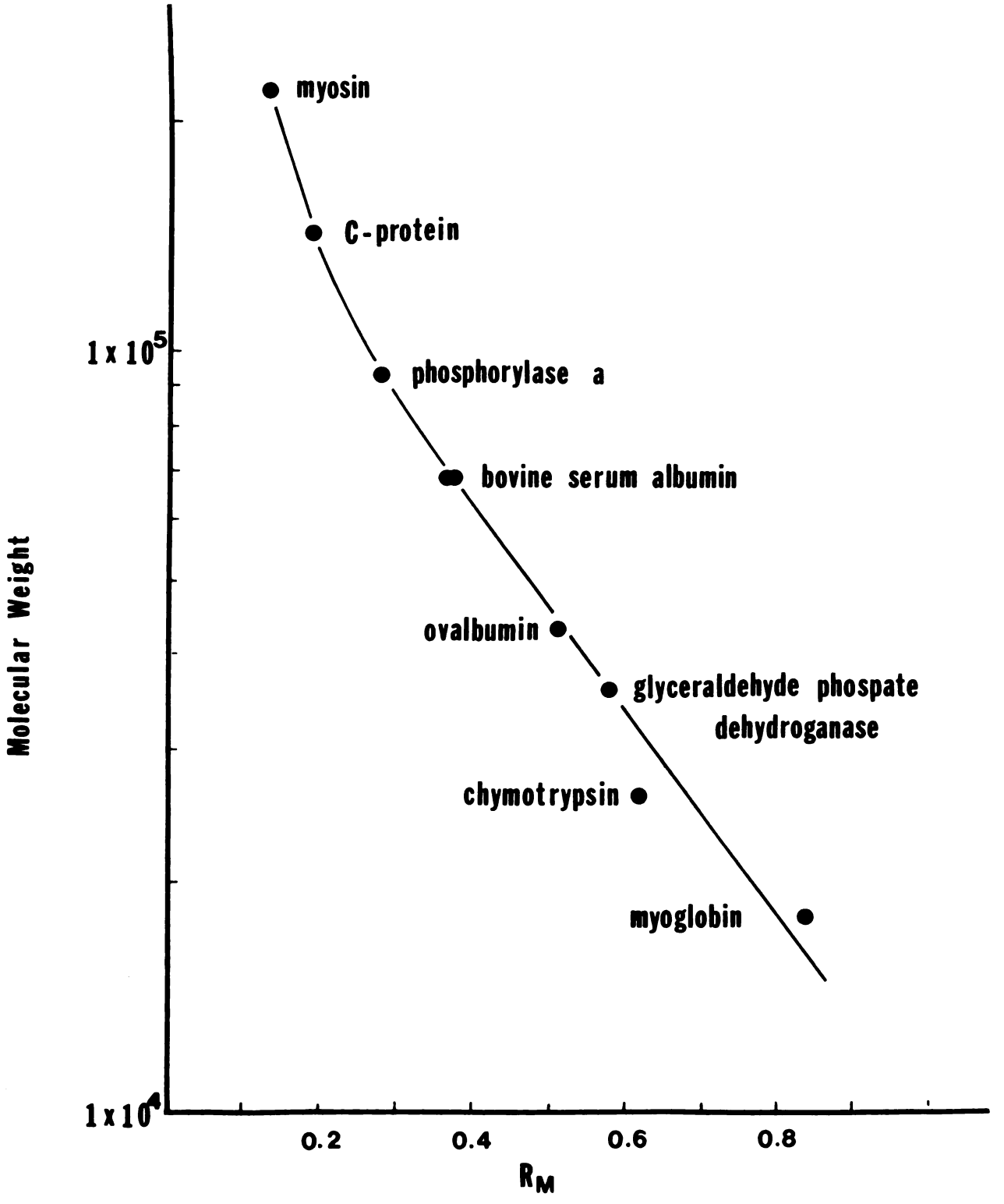


Figure 12. The standard curve for molecular weight estimation using 7.5% acrylamide SDS gels with 0.15% cross-linking.

in rabbit sarcoplasmic reticulum, cannot be found in the SDS gel pattern of beef sarcoplasmic reticulum. Instead of these two proteins found in rabbit sarcoplasmic reticulum, two proteins with molecular weights of 63,000 and 58,000 were found in beef sarcoplasmic reticulum. Although the 63,000 and 58,000 molecular weight proteins did not have the same Rm values as those of 68,000 and 54,000 molecular weight proteins found in rabbit sarcoplasmic reticulum, it is still possible that they are essentially the same. Further work is needed to investigate whether or not the 63,000 and 58,000 proteins have the Ca^{2+} accumulating ability.

Electron Microscopic Study of the Beef Sarcoplasmic Reticulum

Electron micrographs of the sarcoplasmic reticulum from fresh and cold shortened beef sternomandibularis muscle are shown in Figures 13- 15. Figures 13 and 14 show negatively stained fresh and cold shortened sarcoplasmic reticulum vesicles respectively, while Figure 15 shows electron micrographs of beef sarcoplasmic reticulum vesicles from fresh muscle. These pictures clearly show that sarcoplasmic reticulum preparation consists of vesicles ranging from 60 to 600 μm in diameter. However, no difference was observed between the sarcoplasmic reticulum vesicles from fresh and from cold shortened muscle. This means that cold shortening does not affect the size or conformation of sarcoplasmic reticulum vesicles.

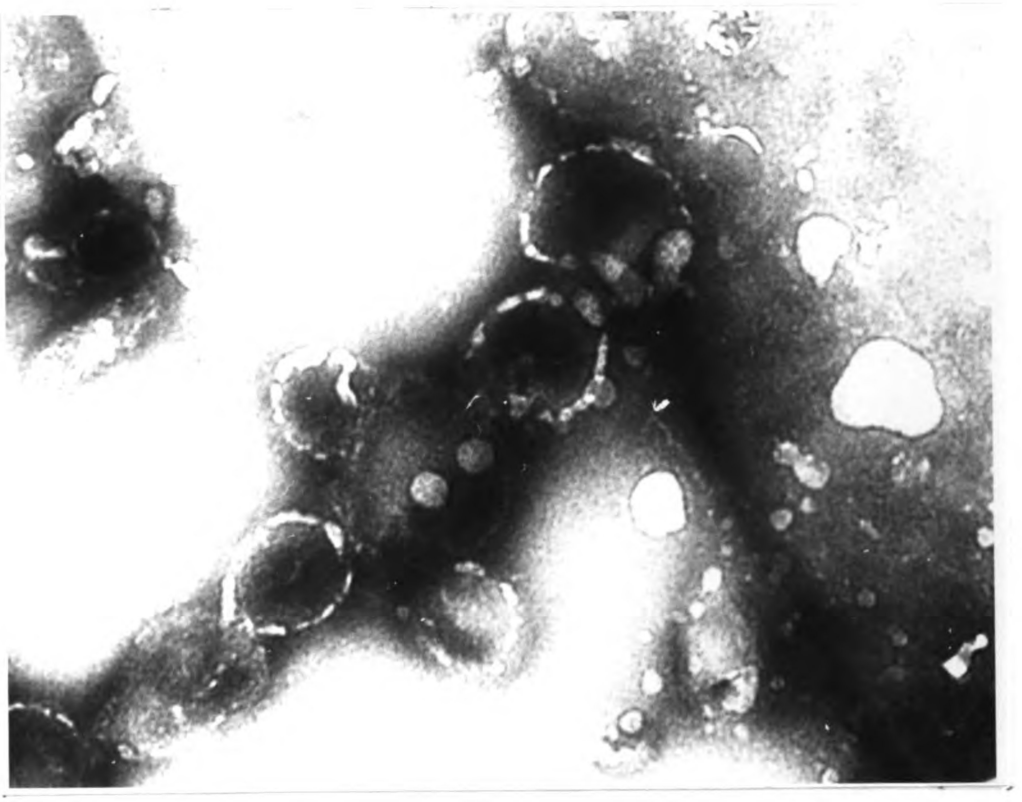


Figure 13. Electron micrograph showing negatively stained fresh beef muscle sarcoplasmic reticulum X 200,000.

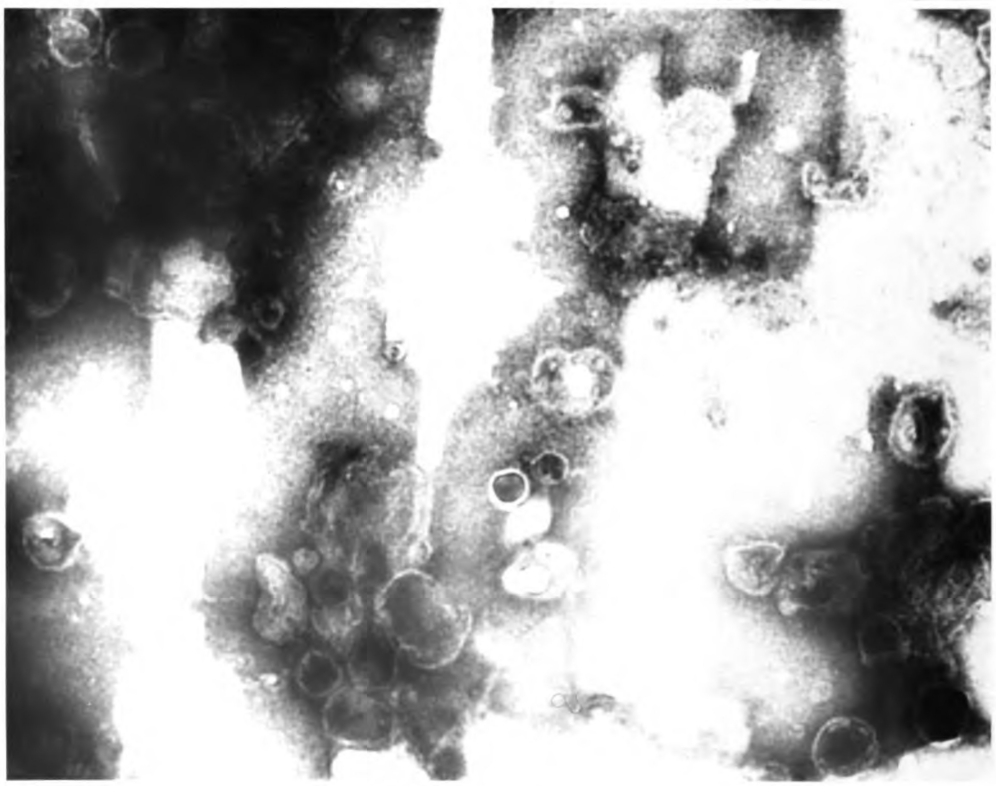


Figure 14. Electron micrograph showing negatively stained cold shortened beef muscle sarcoplasmic reticulum X 160,000.

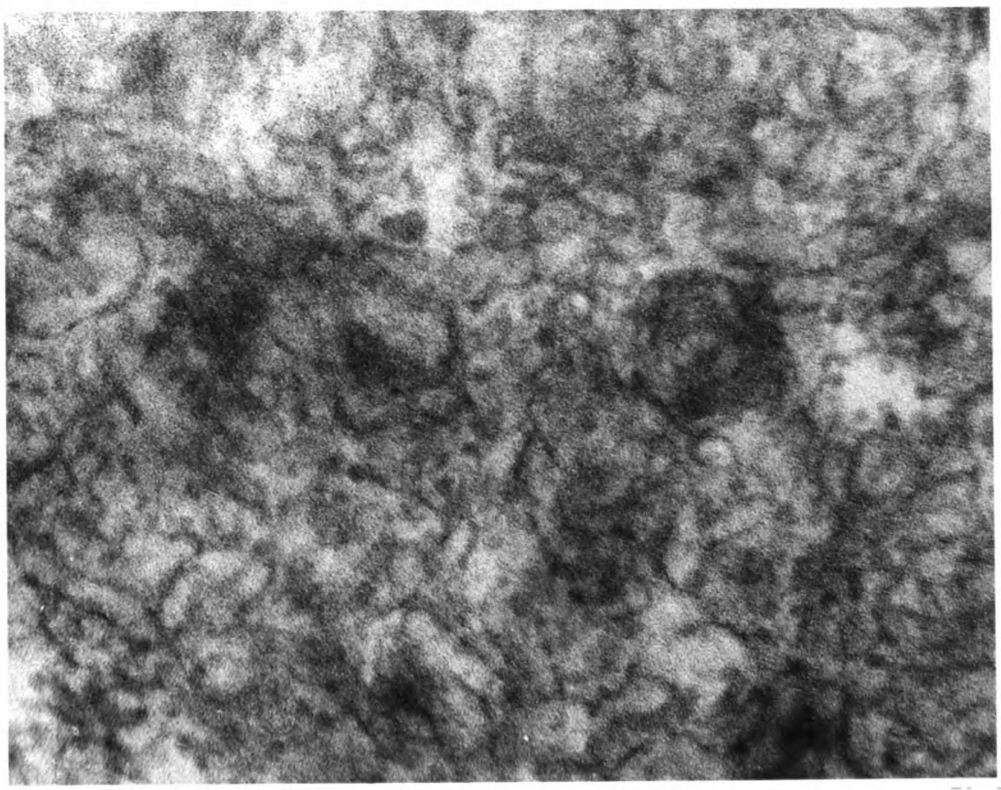


Figure 15. Electron micrograph showing thin sectioned fresh muscle sarcoplasmic reticulum X 125,000.

SUMMARY

Ca^{2+} accumulation and Ca^{2+} release by the sarcoplasmic reticulum vesicles isolated from fresh beef sternomandibularis muscle (immediately after slaughter) and from the muscle held for 24 hours at either 0 or 15°C were determined at several pH values and temperatures. Sarcoplasmic reticulum vesicles were prepared by homogenization of the muscle followed by differential centrifugation with purification by sucrose density gradient centrifugation. Enzyme tests for monitoring the purity of the sarcoplasmic reticulum vesicles proved that they were relatively pure with only slight contamination from the mitochondrial membrane and the lysosomes, and negligible contamination from the sarcolemma.

Ca^{2+} accumulation of the sarcoplasmic reticulum vesicles from fresh and cold shortened (stored for 24 hours at 0°C) muscle was 51 $\pm$ 2.6 and 39 $\pm$ 1.3 nM per mg of protein, respectively, during a 3 minute reaction time at 38°C and pH 7.3. On the other hand, sarcoplasmic reticulum vesicles from muscle stored for 24 hours at 15°C lost all of their activity under the same conditions. The Ca^{2+} accumulating ability of fresh muscle sarcoplasmic reticulum vesicles

decreased with decreasing pH values (7.3, 6.8, 6.2, 5.5 and 5.0) at all temperatures (0, 15 and 38°C). At pH 5.0, sarcoplasmic reticulum vesicles accumulated about 10 nM of Ca^{2+} per mg protein regardless of temperature. This indicates that temperature had no effect upon Ca^{2+} accumulation at pH 5.0. Maximum accumulation of Ca^{2+} (about 50 nM) was observed at 38°C and a pH of 7.3.

Holding sarcoplasmic reticulum vesicles for 10 minutes at pH 7.3 and 38°C greatly decreased the accumulation of Ca^{2+}, resulting in the release of 50% of the total accumulated Ca^{2+}. Twenty nM of Ca^{2+} per mg of protein or a loss of 48% of the total accumulated Ca^{2+} was released by changing the temperature from 38 to 0°C at pH 6.6. On the other hand, lowering the temperature from 38 to 15°C resulted in the release of only 5 nM or about 12% of the total accumulated Ca^{2+} at the same pH. Results, therefore, indicate that low temperatures cause a much greater amount of Ca^{2+} to be released by the sarcoplasmic reticulum.

The effect of simultaneously lowering the pH below 7.3 and the temperature below 38°C were much less effective on Ca^{2+} release than lowering temperature and pH independently. Approximately 10 nM of Ca^{2+} per mg protein or about 25% of the total accumulated Ca^{2+} was released upon simultaneously lowering the pH from 7.3 to 6.6 and the temperature from 38 to 0°C. The results of the present study indicate that pH and temperature treatment altered the Ca^{2+} accumulating ability of the sarcoplasmic reticulum and

caused the release of Ca^{2+} from Ca^{2+}-saturated sarcoplasmic reticulum. Further work is needed to demonstrate whether or not the amount of released Ca^{2+} observed in this study is enough to bring about muscle shortening.

SDS gel electrophoresis of the purified beef sarcoplasmic reticulum gave four major protein bonds, which corresponded to molecular weights of 100,000, 63,000, 58,000 and less than 10,000 daltons. The 100,000 molecular weight protein appeared to be the Ca^{2+}-activated ATPase, which has been identified in rabbit sarcoplasmic reticulum. The 63,000 and 58,000 molecular weight proteins were not found on the SDS gel electrophoresis pattern for purified rabbit sarcoplasmic reticulum. They did not have the same Rm values and molecular weights as Calsequestrin and the high affinity Ca^{2+}-binding protein, which have been found in rabbit sarcoplasmic reticulum. Further work is thus needed to prove whether or not they perform the same function in beef sarcoplasmic reticulum.

Electron microscopic study on fresh and cold shortened muscle revealed no differences in size and conformation. Electron microscopic observations indicate that cold shortening does not affect appreciable changes in the size or conformation of the sarcoplasmic reticulum vesicles.

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APPENDIX

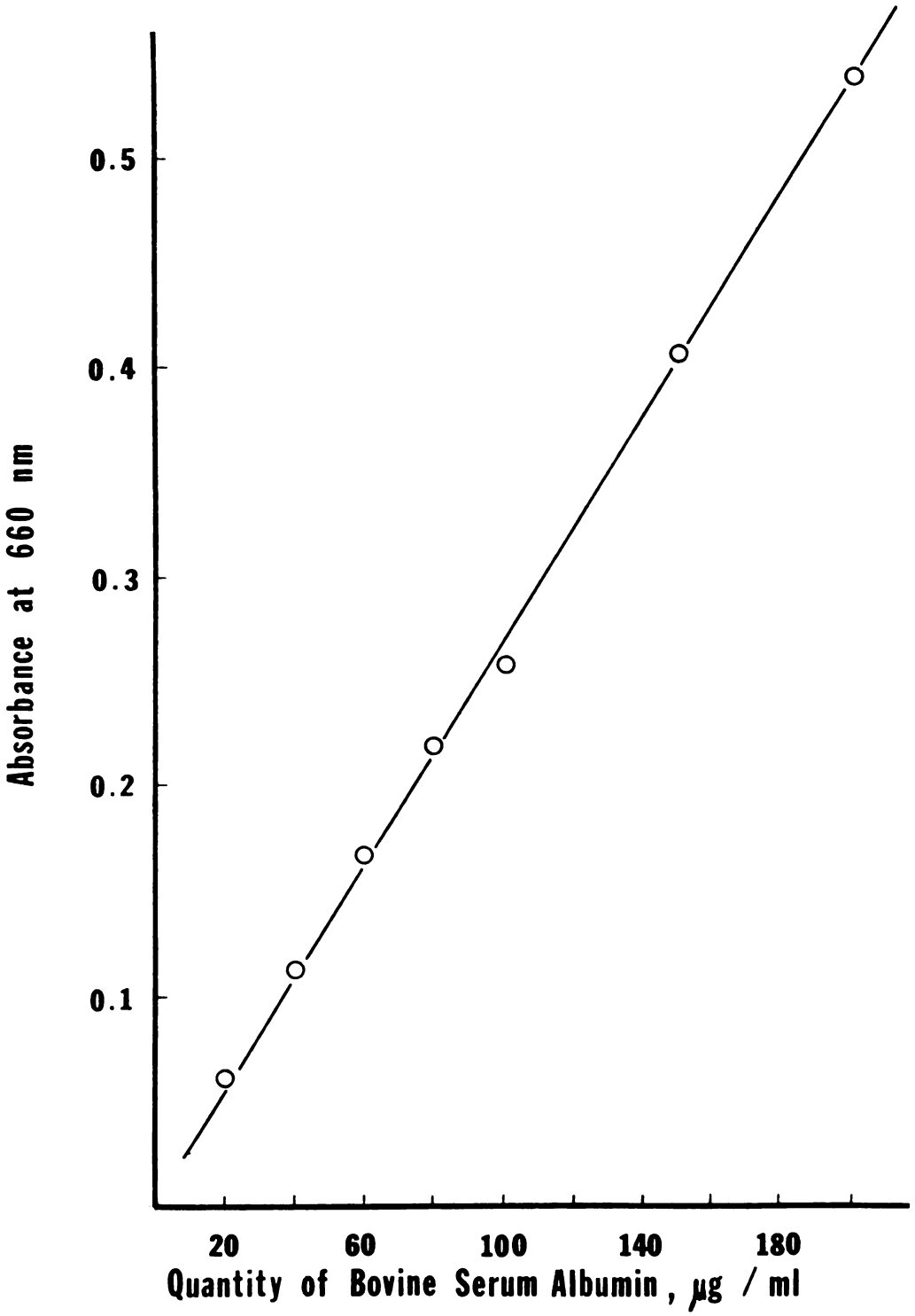

Figure 1. Standard curve for determining protein using Lowry's method.

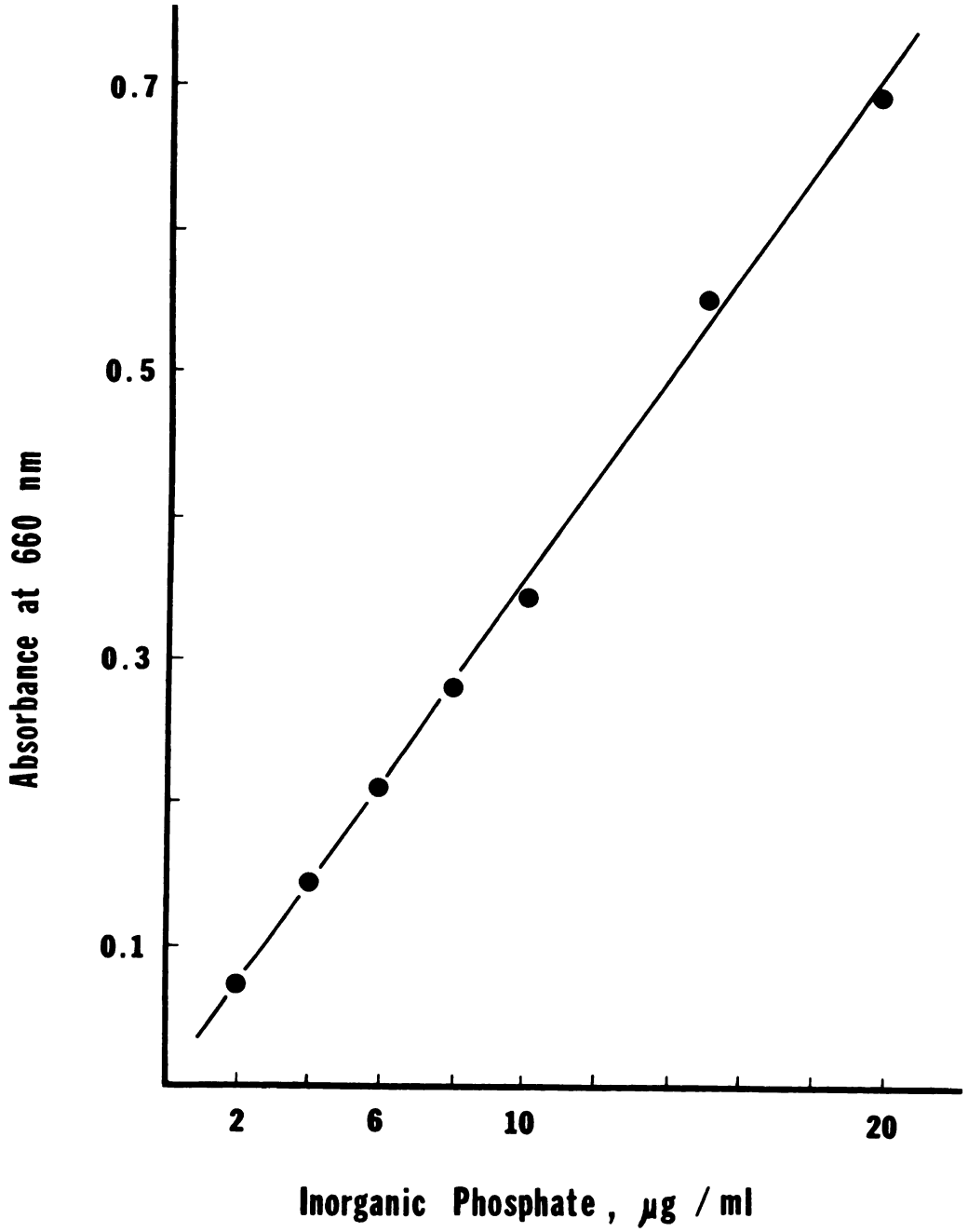

Figure 2. Standard curve for inorganic phosphate determination using Fiske and SubbaRow's method.

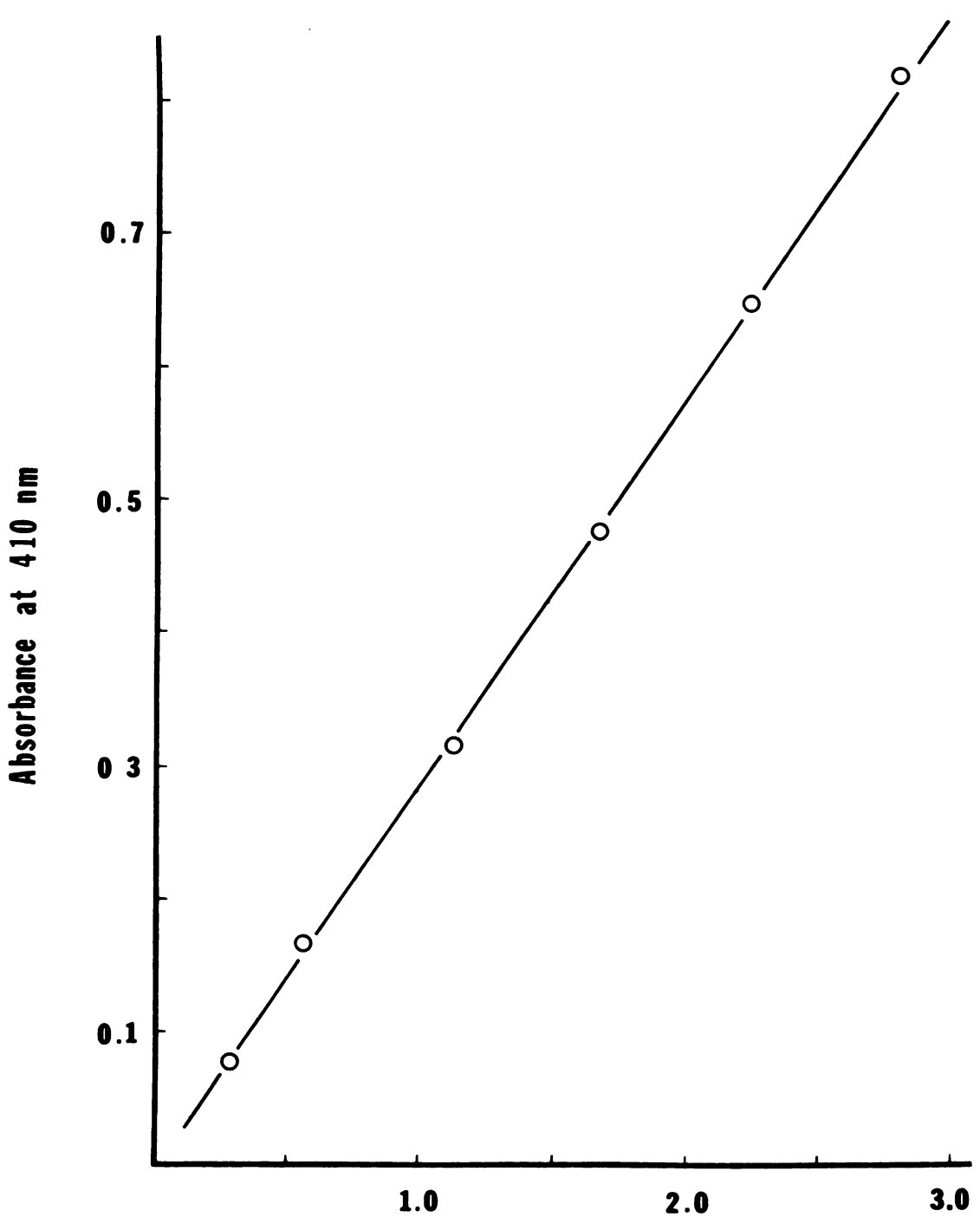

Figure 3. Standard curve for determining acid phosphatase activity (cited from SIGMA Technical Bulletin).