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Purification and properties of skeletal muscle microsomes

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PURIFICATION AND PROPERTIES OF
SKELETAL MUSCLE MICROSONES.

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PURIFICATION AND PROPERTIES OF SKELETAL MUSCLE MICROSONES

by

Bruce Alan Eason

A Dissertation Submitted to the
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ABBREVIATIONS AND SYMBOLS

Å = angstrom = 10^-8 centimeters
ADP = adenosinediphosphate
ATP = adenosinetriphosphate
ATPase = adenosinetriphosphatase
45Ca = radioactive calcium-45
cm = centimeter
EDTA = ethylenediamine-tetraacetate
g = acceleration due to gravity = 980 cm/sec
hr = hour
Hepes = N-2-hydroxethyl piperazine N'-2-ethanesulfonate
K2C2O4 = potassium oxalate
kcal = kilocalorie
μ = micro or micron
M = concentration in moles/liter
min = minute
mg = milligram
ml = milliliter
mM = concentration in millimoles/liter
msec = millisecond
Pi = inorganic phosphate
PPO = 2,5 diphenyloxazole
S = Svedberg, a unit of sedimentation velocity = 10^-13 sec
S.E. = Standard Error of Mean
sec = second
TCA = trichloroacetic acid
Tris = tris-(hydroxymethyl)-aminomethane
INTRODUCTION

Two major objectives of research in cellular biochemistry have been elucidation of the molecular architecture of subcellular organelles and relation of this architecture to biological function. Research toward these objectives has proceeded along two main lines of investigation (a) a morphological approach involving structural characterization of subcellular organelles and in situ localization of cellular reactions by means of histochemistry, and (b) a biochemical approach involving in vitro analysis of isolated subcellular components.

Both these approaches have been useful in the study of membranous structures of the sarcoplasmic reticulum. The sarcoplasmic reticulum is believed to be involved in at least two ways in the contraction--relaxation cycle of muscle. First, the sarcotubular membranes, in response to the muscle cell action potential, are thought to release Ca$^{++}$ to the ionic milieu surrounding the myofibrillar proteins, thereby initiating the contractile interaction between actin and myosin. Second, it is known that the microsomal fraction isolated from muscle is able, under a variety of in vitro conditions, to vigorously accumulate or bind Ca$^{++}$ from the surrounding medium at the expense of a high energy phosphate bond. Projected to the muscle cell, this removal of Ca$^{++}$ from the environment surrounding the myofibrils is thought to effect relaxation of muscle.

Simple sedimentation methods (differential centrifugation) have been used to isolate sarcotubular fragments from muscle, and these isolated fragments have, in turn, been used for in vitro studies of the biochemical properties of the sarcotubular Ca$^{++}$-sequestration system. Although these
sarcotubular preparations have been very useful in elucidation of the role of $\text{Ca}^{++}$ in muscle contraction, a review of the literature indicates that some of these preparations contain not only sarcotubular membranes, but also other cytological debris. This causes considerable ambiguity in interpreting results of these *in vitro* studies and also makes it impossible to quantitate $\text{Ca}^{++}$-binding in terms of moles of $\text{Ca}^{++}$ bound per unit of sarcotubular membrane protein. Use of high speed density gradient and zonal centrifugation has greatly increased the resolution and purity obtainable in subcellular preparations. However, there have been very few systematic studies either of the purity of sarcotubular vesicle preparations or of how to obtain maximal purity of such preparations. Indeed, density gradient centrifugation has not been very widely used for purification of muscle microsomes. One of the reported studies which did use density gradient centrifugation for purification found that some heterogeneity exists, in ordinary density gradient-purified muscle microsomal preparations.

Most of the work on the muscle microsomal fraction has been directed toward elucidation of its function in living muscle. Very little information has been obtained about post-mortem changes in the activity of muscle microsomes. According to Nauss and Davies (1966), the onset of *rigor mortis* in post-mortem muscle may be triggered by an efflux of $\text{Ca}^{++}$ from damaged sarcoplasmic reticulum.

Since several reports have questioned the purity of skeletal muscle microsomal fractions and since there is limited information about the post-mortem behavior of skeletal muscle microsomes, the present investigation was undertaken (a) to re-examine the purity of muscle microsomal
preparations and to develop an improved procedure for the isolation and purification of skeletal muscle microsomes, and (b) to examine microsomes isolated from skeletal muscle homogenates for loss of functional integrity before, during, and after the development of rigor mortis in muscle.

Limitations of This Study

Muscle is a highly ordered and complex system, but homogenization causes considerable disruption of this highly structured array. Thus, not only are the sarcoplasmic reticular membranes broken, but also the membranes of the sarcolemma, the mitochondria, nucleus, etc. These membranes all have a lipoprotein composition, and it is conceivable that membrane fragments other than sarcotubular membranes might sediment in microsomal preparations. In the electron microscope, these other membranes might appear as vesicles, similar in appearance to the sarcoplasmic reticular vesicles. Furthermore, due to their lipoprotein composition, these membranes are all insoluble in aqueous systems. Because of these similarities, it is very difficult to detect contaminating membrane fragments in sarcoplasmic reticular preparations, and even more intractable to eliminate such contamination once it is detected.

In addition to the preceding difficulties, there is some inherent difficulty with use of the word "pure". It is virtually impossible to prove that a new protein or a subcellular organelle is pure in the strict sense of the word. Thus, application of new and more sensitive techniques has frequently led to the appearance of heterogeneity in supposedly "pure" preparations. It is easier to prove that a substance is heterogeneous than to prove that it is pure. Therefore, this study does not claim to
have devised a method for preparation of "pure" muscle microsomes, but rather, contends that the preparative techniques developed will produce a sarcotubular vesicle preparation with a higher specific ATPase activity and a faster rate of Ca$^{++}$-accumulation than preparative techniques currently in use.

Because of the almost infinite variety of possible approaches to purification of muscle microsomes, it was not feasible to examine all possible solvents and gradient systems in this study. Rather, the commonly-used sucrose solutions were adopted as the basic medium for most of the purification attempts reported here. Also, because of the small yield of purified microsomes and the time involved in development of a satisfactory purification procedure, it was impossible to study the effects of a wide variety of activators, ionic strengths, pH values, or temperatures on the ATPase and Ca$^{++}$-sequestering activities of the purified microsomes.

Results from the Ca$^{++}$-sequestration studies may be overestimates of the real ability of the microsomal fraction to accumulate calcium, since it is well known that oxalate potentiates Ca$^{++}$-accumulating ability of muscle microsomes. However, Ca$^{++}$-sequestration is low and variable in the absence of oxalate. Therefore, oxalate was included in all Ca$^{++}$-sequestration assays reported in this study because the presence of oxalate results in a reliable and sensitive assay for comparison of Ca$^{++}$-sequestration activities.
In 1948, Kielley and Meyerhof demonstrated that skeletal muscle contained an ATPase activity which appeared to be soluble in low ionic strength extracts, but which could be sedimented by high speed centrifugation. The particulate nature of this ATPase distinguished it from myosin ATPase, one of the other proteins exhibiting ATPase activity in skeletal muscle. Moreover, the particulate ATPase exhibited a pH optimum about pH 6.8 in contrast to the pH optimum of 9.5 exhibited by myosin, and was activated by Mg$^{++}$ and to a lesser extent by Mn$^{++}$, whereas myosin is potently inhibited by Mg$^{++}$. Also, Ca$^{++}$, Ba$^{++}$, Co$^{++}$ ions had very little effect on the ATPase activity of the particulate fraction, although Ca$^{++}$ is a strong activator of either myosin or actomyosin ATPase activity.

Further studies showed that the particulate ATPase was inhibited by 5 mM Ca$^{++}$ in the presence of 5 mM Mg$^{++}$, by fluoride ions, and by p-chloromercuribenzoate.

Several years later, Marsh (1951, 1952) found that fiber volume in homogenates of at-death muscle would undergo a remarkable decrease after several hr of post-mortem storage. In some cases, the volume of the shrunken fibers could be increased by addition of fresh ATP, but in other cases, addition of ATP caused an additional volume decrease resembling synaeresis of purified myofibrils. It was found that response to ATP addition was governed by the presence of a factor in the supernatant solution of a centrifuged (1750 x g) muscle homogenate. In the absence of this "sarcoplasmic factor", ATP addition caused only synaeresis of the shrunken fibers, but in the presence of the "sarcoplasmic factor" ATP
addition caused a fiber volume increase. The "sarcoplasmic factor" was very labile and could ostensibly be inhibited by addition of Ca++. Furthermore, it was found that the Mg++-modified ATPase activity of muscle fibrils was suppressed by the presence of the "sarcoplasmic factor".

Marsh's discovery evoked a great deal of interest among muscle biochemists, and because of the suspected connection between fiber volume increase and muscle relaxation, Marsh's "sarcoplasmic factor" soon became known as "Marsh's relaxing factor" or simply the "relaxing factor". It was shown that the relaxing factor will relax (lengthen) contracted muscle fiber bundles in the presence of Mg++ and ATP (Bendall, 1952, 1953). In addition, the relaxing factor will inhibit synaeresis and ATPase activity of well-washed myofibril preparations (Hasselbach and Weber, 1953). In the succeeding years, "Marsh's relaxing factor" was variously identified as myokinase (Bendall, 1954), a phosphocreatine-creatine phosphokinase system (Goodall and Szent-Györgyi, 1953; Lorand, 1953), pyruvate phosphokinase (Moos and Lorand, 1957), phosphoenol-pyruvate (Lorand et al., 1958) and an α-glycerol phosphate, Mg++, and P_1 system (Marsh, 1960). However, it later developed that none of these substances were the "relaxing factor" of Marsh, but instead, exhibited relaxing activity because they all could contribute to resynthesis of ATP from ADP, either alone or in combination with contaminants present in the myofibril systems normally studied.

The discovery which eventually led to identification of "Marsh's relaxing factor" was the observation by Kumagai et al. (1955) that the Kielley-Meyerhof microsomal ATPase preparation possessed relaxing activity. This discovery was subsequently confirmed and extended by Ebashi (1958).
Soon after Kumagai's observation, Portzehl (1957) showed that relaxing activity was sedimentable and was therefore associated with a particulate fraction in muscle. Previous efforts to identify the relaxing factor had been unsuccessful in part because investigators had been seeking a soluble protein to identify as the "relaxing factor". Portzehl's work showed that 100% of the relaxing factor activity could be sedimented between 6,000 x g for 20 min and 34,000 x g for 60 min.

Concomitantly with recognition of the particulate nature of the relaxing factor, evidence began to accumulate which suggested that the ultrastructural component called the sarcoplasmic reticulum was actively involved in contraction. Porter and Palade (1957) "rediscovered" the sarcoplasmic reticulum in muscle cells and provided an ultrastructural description of this membranous organelle. The earlier light microscopic descriptions of this organelle by Veratti (1902) had been overlooked for 55 years. Porter and Palade's work, together with many other more recent studies, has shown that the sarcoplasmic reticulum consists of a fine network of membrane-limited tubules. This network can be divided into two morphologically distinct components, the T- or transverse system and the L- or longitudinal system. The T- system consists of a series of larger tubules extending perpendicularly from the sarcolemma inward toward the center of the fiber at regular intervals along the length of the fiber. The longitudinal system consists of a series of smaller tubules extending in both directions from the T-tubules and enveloping the myofibrils as lace-like sleeves. As these smaller tubules approach the T-tubules, they join to form large lateral cisternae adjacent the T-tubules. When sectioned in the proper plane and examined in the electron microscope,
the T-tubules with their associated cisternae on both sides appear as three, electron-translucent openings which has been referred to as a "triad". It has been shown that the lateral cisternae do not communicate directly with the T-tubules and the T- and L- systems are separate sets of membrane-enclosed tubules.

A. F. Huxley and coworkers (Huxley and Straub, 1958; Huxley and Taylor, 1958; Huxley, 1959) found that local depolarization of a very small area of the sarcolemmal membrane produces a local contraction only when this depolarization is done at those levels of the sarcomere where the triad structures of the sarcoplasmic reticulum are located. These triads dependent upon the species are situated at different locations in the A and I bands of striated muscles. It was concluded that membranes of the triad must be able to conduct impulses inward from the sarcolemma to the interior of the muscle cell. This conclusion has since been substantiated by several lines of evidence (Constantin and Podolsky, 1966, 1967), and it has now been demonstrated that the central tubules of the triad are, in fact, invaginations of the sarcolemma itself (Franzini-Armstrong and Porter, 1964; Huxley, 1964; Page, 1964). It was clear from this early work of A. F. Huxley that the sarcoplasmic reticulum must have the ability to interfere, or to produce a substance which would interfere, with the contractile interaction among ATP, actin, myosin and Mg++. Soon after implication of the sarcoplasmic reticulum in the contractile process, it was suggested that the relaxing factor, a chemical entity, was in fact identical with the sarcoplasmic reticulum, a morphological entity (Nagai et al., 1960; Porter, 1961; Muscatello et al., 1961, 1962; Ebashi, 1960; Ebashi and Lipmann, 1962). Electron micrographs of the
particulate fraction of muscle sedimented between 8,000 x g for 20 min and 25,000 x g for 60 min showed that this fraction was vesicular and tubular-like in nature (Nagai et al., 1960). The diameter of the spherical or ellipitical vesicles was 600 - 3,000 Å and oftentimes elongated tubules 150 - 300 Å in diameter could be seen. The limiting membranes of both the vesicles and elongated tubules were structurally very similar to membranes of the sarcoplasmic reticulum. Ebashi and Lipmann (1962) even found some complete triad structures in a muscle microsomal fraction that was sedimented between 10,400 x g for 20 min and 38,000 x g for 60 min. Homogenization of muscle obviously results in disintegration of the organized structure of the sarcoplasmic reticulum, so the isolated vesicles in the particulate relaxing factor preparation must consist of resealed fragments of the sarcoplasmic reticular membranes. Therefore, it has been proposed that after their disruption by homogenization, the sarcotubular membranes must undergo a healing process similar to that which occurs at the injured surface membrane of a heart muscle fiber. All evidence available thus far suggests that the particulate relaxing factor activity is associated only with membranous elements derived from the sarcoplasmic reticulum and not with mitochondrial membrane fragments nor with other microsomal membranous elements.

Although the relaxing factor activity could be associated with the sarcoplasmic reticulum, this association still did not afford any revealing insight into the mechanism of action of the relaxing factor. This insight was soon provided largely through the efforts of Hasselbach, Ebashi, A. Weber and their coworkers (Ebashi, 1960, 1961; Ebashi and Ebashi, 1962; Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961; Weber, 1959;
Weber and Winicur, 1961; Weber and Herz, 1963). Briefly, Weber and her associates demonstrated that natural actomyosin (actomyosin prepared by a 24-hour extraction of minced muscle) or myofibrils at ionic strengths of 0.1 - 0.16 required trace amounts ($10^{-7}$ to $10^{-5}$ M) of Ca$^{++}$ for maximal Mg$^{++}$-modified ATPase activity or for synaeresis. High Mg$^{++}$-modified ATPase activity and synaeresis are considered in vitro analogues of contraction in such systems, whereas low Mg$^{++}$-modified ATPase activity and inhibition of synaeresis in the presence of ATP and Mg$^{++}$ are considered in vitro analogues of relaxation. Concurrently with Weber's work, Ebashi (1960, 1961) showed that the particulate relaxing factor possessed a strong affinity for Ca$^{++}$ when incubated in an in vitro system containing Mg$^{++}$ and ATP. Furthermore, it was soon shown that the particulate relaxing factor would remove Ca$^{++}$ which was bound to actomyosin when both were incubated together in an in vitro system (Ebashi, 1961). These findings were independently confirmed and extended by Hasselbach and Makinose (1961).

A large number of papers substantiating Ebashi's and Weber's early work have appeared in the last six to seven years (Baltscheffsky, 1964; Carsten and Mommaerts, 1964; Ebashi and Yamanouchi, 1964; Elison et al., 1965; Fanburg and Gergely, 1965; Fanburg et al., 1964; Harigaya et al., 1968; Hasselbach and Makinose, 1963; Inesi and Watanabe, 1967; Lee et al., 1965; Makinose and Hasselbach, 1965; Makinose and The, 1965; Martonosi and Feretos, 1964a, 1964b; Pease et al., 1965; Schuel et al., 1965; Seidel and Gergely, 1964; Szabolcs and Köver, 1965; and Weber et al., 1963, 1966), and it now seems certain that muscle contraction is initiated and stopped by regulation of free intracellular Ca$^{++}$-levels, mediated by sequestration
and release of Ca\(^{++}\) from the membranes of the sarcoplasmic reticulum. Four excellent reviews have been published summarizing the evidence concerning the nature of the relaxing factor (Hasselbach, 1964b; Sandow, 1965; Smith, 1966; and Weber, 1966).

Although it was possible to demonstrate that trace amounts of Ca\(^{++}\) were necessary for contraction in myofibrillar or natural actomyosin systems, synthetic actomyosin systems made by mixing purified actin and purified myosin were totally insensitive to the presence of Ca\(^{++}\). Thus, synthetic actomyosin systems possess a high Mg\(^{++}\)-modified ATPase activity and exhibit synaeresis even when Ca\(^{++}\) concentrations were \(10^{-8}\) M or less. This difference between synthetic actomyosin and natural actomyosin or myofibrils was resolved when Ebashi and Ebashi (1964) discovered a new myofibrillar protein factor, present in myofibrils and natural actomyosin but missing from synthetic actomyosin. This new protein factor acted to sensitize actomyosin to Ca\(^{++}\). Thus, synthetic actomyosin in the absence of this new protein factor would undergo synaeresis at very low Ca\(^{++}\) concentration, but in the presence of this new protein factor, \(10^{-6}\) to \(10^{-5}\) M Ca\(^{++}\) was required for maximal Mg\(^{++}\)-modified ATPase activity or synaeresis. Because of similarities between the properties of this new protein factor and the properties of tropomyosin, Ebashi and Ebashi (1964) called their new protein factor "native tropomyosin". In the rapid developments that have followed Ebashi's discovery, it has been found that native tropomyosin is in fact a mixture of two proteins, tropomyosin itself, and a new protein called troponin (Ebashi and Kodama, 1965). Troponin is the actual Ca\(^{++}\)-sensitizing protein, but purified troponin does not bind to either
myosin or actin and is therefore ineffective alone (Ebashi et al., 1967; Komizu and Maruyama, 1967). Tropomyosin interacts strongly with troponin and since tropomyosin also binds strongly to F-actin, it is now generally felt that tropomyosin acts as a "cement", serving to bind troponin to the thin or actin filaments (Ebashi, 1963; Ebashi et al., 1968). It is known that both tropomyosin and troponin together are necessary for the Ca\(^{++}\)-sensitizing effect, and that neither of these proteins alone is effective. Recent developments have shown that the Ca\(^{++}\) released by the sarcoplasmic reticulum to initiate contraction is bound by troponin (Fuchs and Briggs, 1968), and that this Ca\(^{++}\) is then removed from troponin to effect relaxation. However, the mechanism whereby binding of Ca\(^{++}\) to troponin can trigger contraction is entirely unknown. Ebashi et al. (1968) have stated that Ca\(^{++}\) causes depolymerization of troponin in purified solutions, but further information on the triggering effect of Ca\(^{++}\) is lacking.

The discovery of troponin and its role in muscle now makes it appear virtually certain that muscle contraction is initiated and stopped by regulation of free intracellular Ca\(^{++}\) levels between 10\(^{-7}\) and 10\(^{-5}\) M. Therefore, recent efforts have been directed toward understanding the mechanism of Ca\(^{++}\) sequestration by the sarcotubular membranes, and trying to localize the sites of Ca\(^{++}\)-binding. Hasselbach and Makinose (1961, 1963) have shown that ATP is necessary for Ca\(^{++}\) sequestration by fragmented sarcoplasmic reticulum. These workers suggested that Ca\(^{++}\) sequestration is an active transport process and showed (Hasselbach, 1964b) that Ca\(^{++}\)-uptake is coupled to a Mg\(^{++}\)-activated ATPase activity in fragmented sarcoplasmic reticulum. The presence of 10\(^{-7}\) - 10\(^{-5}\) M Ca\(^{++}\) caused an activation of the Mg\(^{++}\)-modified ATPase activity of fragmented
sarcoplasmic reticulum ("extra-splittings"), this activation being coupled to Ca^{++}-uptake. In terms of Hasselbach's concept, therefore, Ca^{++} is transported across the sarcoplasmic reticular membrane into the inner part of the tubule, where it is retained in free form against an electrochemical gradient. The presence of oxalate in in vitro assays of Ca^{++}-sequestration greatly increases total Ca^{++} uptake by vesicles presumably because Ca-oxalate is precipitated inside the vesicles.

In a series of recent papers, however, Carvalho (Carvalho and Leo, 1967; Carvalho, 1968a, 1968b) has proposed that Ca^{++} is bound to fixed binding sites on the surface of the sarcoplasmic reticular membranes. These binding sites also interact with other cations such as Mg^{++}, K^{+}, and H^{+}. The amount of each cation bound depends on the relative affinity of the sites for each cation and the relative concentrations of the cations in the medium. According to Carvalho, the role of ATP in Ca^{++}-binding is to induce a conformational change in the membrane protein; this conformational change results in a selective binding of Ca^{++} and a displacement of K^{+}, Mg^{++}, and H^{+}. Carvalho's proposal for the mechanism of Ca^{++}-uptake by sarcoplasmic reticulum is supported by Winegrad's autoradiographic experiments showing that during recovery of frog muscles from tetanus, relaxation occurred as a result of Ca^{++} binding at the outer surface of the sarcoplasmic reticular membrane rather than transport of Ca^{++} across the membrane (Winegrad, 1968). Inesi and Watanabe (1967) also found that in the absence of oxalate, a significant proportion of total Ca^{++} taken up is simply bound to the membrane. However, in the presence of oxalate, the amount of Ca^{++} bound to the membranes is very small compared to total Ca^{++}-uptake. Inesi and Watanabe suggested that in the
Presence of oxalate, Ca\(^{++}\) is precipitated as Ca-oxalate inside the vesicles. This lowers the intravesicular concentration of free Ca\(^{++}\), and the release of Ca\(^{++}\) ions inside the vesicles continues as long as external Ca\(^{++}\) is available. In the absence of oxalate, an increased intravesicular concentration of free Ca\(^{++}\) prevents further release of Ca\(^{++}\) inside the vesicles and Ca\(^{++}\) bound to the exterior of the membranes represents a sizable portion of total Ca\(^{++}\)-uptake. Present evidence does not permit an unequivocal choice as to whether the binding or the transport mechanism of Ca\(^{++}\)-sequestration is the mechanism operating in vitro. However, it is clear that a sizable proportion of sequestered Ca\(^{++}\) (80% according to Carvalho) is bound to the sarcoplasmic reticular membranes (either on the inside or outside) and does not contribute to total activity of the cation accumulated.

Several other properties of the sarcotubular membrane have been examined in an effort to determine the mechanism of Ca\(^{++}\)-uptake. Mommaerts (1967) found that the circular dichroic spectrum of microsomes actively transporting Ca\(^{++}\) was the same as that of inactive microsomes. This would seem to rule out conformational changes of membrane proteins as a factor in Ca\(^{++}\)-sequestration. However, on the basis of indirect evidence, Weber (1968) has suggested that the Ca\(^{++}\)-sequestration system of fragmented sarcoplasmic reticulum exists in two different conformational states. Inesi and Watanabe (1967) found that the temperature dependence of both Ca\(^{++}\)-uptake and the Mg\(^{++}\)-modified ATPase activity differed in the presence and absence of oxalate. In the presence of oxalate, the energy of activation for Ca\(^{++}\)-uptake increased from 13 - 16 kcal/mole to 20 - 30 kcal/mole and the energy of activation for Mg\(^{++}\)-modified ATPase activity increased.
from 14 - 16 kcal/mole to 23 - 27 kcal/mole.

Although the mechanism of Ca\(^{++}\)-sequestration remains unclear, both the phospholipid and protein components of sarcotubular membranes are necessary for Ca\(^{++}\)-accumulation. Martonosi et al. (1968) found that extensive digestion by phospholipase C destroys the Ca\(^{++}\)-sequestration ability and inhibits the Mg\(^{++}\)-Ca\(^{++}\)-modified ATPase activity of sarcoplasmic reticular membranes. Addition of purified lecithin to the phospholipase C-digested membranes restored much of their original ATPase activity and ability to sequester Ca\(^{++}\). Inesi and Asai (1968), Ikemoto et al. (1968), and Martonosi (1968a) found that very brief (5 - 10 min) digestion with trypsin causes an increase in the Mg\(^{++}\)-modified ATPase activity, but a marked decrease in the Ca\(^{++}\)-uptake ability of fragmented sarcoplasmic reticulum. Prolonged digestion times were required to inhibit the ATPase activity of the fragmented membranes, this inhibition occurring only after extensive ultrastructural changes had occurred in the vesicles. Thus, it appears that a few peptide bonds, which are very susceptible to trypsin, are essential to Ca\(^{++}\) sequestration but are not involved in the Mg\(^{++}\)-modified ATPase activity of sarcotubular vesicles. A number of treatments, including aging (Hasselbach, 1964a; Molnar and Lorand, 1962; Sréter and Gergely, 1964), sonication (Diehl et al., 1964; Sréter and Gergely, 1964), acetone (Diehl et al., 1964), ethanol (Molnar and Lorand, 1962), or Na oleate (Hasselbach and Makinose, 1961) will leave the Mg\(^{++}\)-modified ATPase activity unchanged while almost totally inhibiting Ca\(^{++}\)-uptake. It is of interest that phospholipase C digestion appears to destroy ATPase activity and Ca\(^{++}\)-uptake of sarcotubular membranes simultaneously.
Several studies have suggested that Ca\textsuperscript{++}-uptake and storage occur in
different \textit{in vitro} locations of the sarcoplasmic reticulum. Gauthier
(1967) used histochemical techniques at the light microscopic level to
demonstrate four different kinds of ATPase activities in skeletal muscle
fibers of rat diaphragm muscle. Two of these could be associated with the
mitochondrial and myofibrillar ATPase activities, but the other two were
associated with the sarcotubular system. Of the two sarcotubular ATPases,
one was located at the level of the triad and the other was found near the
center of the A-band. Gauthier suggested that the ATPase activity at the
level of the triad was associated with release of Ca\textsuperscript{++} whereas the ATPase
activity near the center of the A-band was associated with rebinding of
Ca\textsuperscript{++} to sarcoplasmic reticular membranes. Thus, in Gauthier's scheme,
Ca\textsuperscript{++} was bound by the fine tubules of the longitudinal system of the
sarcoplasmic reticulum and was then transported along these tubules to
the lateral cisternae, where it was stored until released in response to
a nerve stimulus.

Gauthier's proposal has recently received direct experimental support
from the autoradiographic studies of Winegrad (1968). By studying the
time-course of intracellular Ca\textsuperscript{++} movements in frog skeletal muscle during
recovery from tetanus, Winegrad found that Ca\textsuperscript{++} was rebound to the fine
network of longitudinal tubules and was then transported along these
tubules to the lateral cisternae. Exchange of calcium in resting muscle
occurs primarily between the lateral cisternae and the T-tubules. A shift
of calcium from the region of the lateral cisternae to the immediate
vicinity of the thick filaments occurs upon chemical stimulation.
Thus, present evidence on the nature of the relaxing factor in muscle indicates that the relaxing factor is identical with at least part of the membranous structures of the longitudinal system of the sarcoplasmic reticulum, and that the action of the relaxing factor is mediated through its ability to bind and release Ca\textsuperscript{++} in response to nerve stimuli. However, the mechanism of Ca\textsuperscript{++} binding remains unclear as does the mechanism whereby a nerve stimulus causes release of Ca\textsuperscript{++}. It does appear that Ca\textsuperscript{++}-binding and Ca\textsuperscript{++}-release occur at two different sites on the longitudinal system, the Ca\textsuperscript{++} being bound by the smaller tubules and then transported to the lateral cisternae from whence it is released.

Isolation and Purification of Sarcotubular Vesicles

The technique of differential centrifugation has been used extensively in recent years for isolation of sarcoplasmic reticular fragments from muscle homogenates. The review of Hasselbach (1964b) summarizes procedural details for many of the methods used for preparation of relaxing vesicles from muscle homogenates by differential centrifugation. In general, most workers have selected isotonic or nearly isotonic sucrose or salt solutions, buffered in the range of pH 7.0 - 7.2, for initial homogenization of muscle. A few investigators, however, have shown preference for the use of hypertonic sucrose solutions. Times used for homogenization are quite variable, with some investigators using a very short homogenization time (15 sec) and others using much longer homogenization periods of 2 to 10 minutes. Also, differential centrifugal preparation of active particulate fractions which show relaxing factor activity has usually been done by using centrifugal forces between 6,000 and 105,000 x g and centrifugal times
of 20 to 90 minutes.

In 1963 Hasselbach and Makinose (1963) reported the first attempt to apply sucrose gradient centrifugation to the purification of sarco-tubular vesicles. A microsomal fraction was isolated from rabbit skeletal muscle by homogenization in 0.1 M KCl, 5 mM K$_2$C$_2$O$_4$, containing 5 mM phosphate or histidine buffer, pH 7.0, followed by differential centrifugation between 8,000 x 10 and 25,000 x 60 (g x min). This particulate preparation was further fractionated into four subfractions by centrifuging through a linear sucrose gradient (0.3 → 1.2 M) at 10,000 x g for 1 hour. The top layer isolated from the gradient was the most active fraction in terms of Mg$^{++}$-modified ATPase activity, Ca$^{++}$-uptake, and in inhibition of myofibrillar ATPase activity.

Following Hasselbach's initial attempt, several other reports have appeared which describe techniques for purification of skeletal or cardiac microsomes by use of sucrose density gradient or zonal centrifugation. By using centrifugation at 70,000 x g through a (0.5 → 2.0 M) sucrose gradient, Kinoshita et al. (1964) resolved microsomes from rabbit skeletal muscle into six subfractions. The most active fraction in terms of relaxing factor activity was isolated from a zone distributed near the middle of the gradient tubes. Kinoshita's crude microsomes were prepared by differential centrifugation between 6,000 and 34,000 x g.

Seraydarian and Mommaerts (1965) also used density gradient centrifugation in sucrose for purification of sarcotubular vesicles. This study was more extensive than some earlier studies on purification of muscle microsomes since two crude microsomal fractions, a light fraction isolated
between 57,000 and 198,000 x g and a heavy fraction, isolated between 15,000 and 57,000 x g, were investigated. Rabbit skeletal muscle was homogenized in 0.3 M sucrose, 20 mM Tris, pH 7.4. When layered on a discontinuous (35 → 70%) sucrose gradient, the heavy microsomal fraction was resolved into two layers, both of which were able to accumulate calcium and inhibit myofibrillar ATPase. The upper layer amounted to slightly more than half of the total material recovered and differed from the lower layer in that its Ca\(^{++}\)-sequestering ability was stable during storage for as long as three weeks. The Ca\(^{++}\)-accumulating ability of the lower layer decreased rapidly during storage. Seraydarian and Mommaerts suggested that resolution of the heavy microsomal reaction into two layers by density gradient centrifugation was due to the presence of empty and filled vesicles, with the filled vesicles having a greater density and therefore sedimenting more rapidly through a density gradient. This hypothesis was supported by electron micrographs of the two layers showing a preponderance of vesicles filled with an electron dense material in the lower layer and the presence of empty vesicles in the upper layer. It was suggested that the filled vesicles could accumulate Ca\(^{++}\) but were unable to retain the Ca\(^{++}\) without leakage. This may account for the weaker relaxing activity of the lower layer.

The light microsomal fraction in Seraydarian and Mommaerts' study was separated into three or four zones on a discontinuous (30 → 80%) sucrose gradient. The material in these zones all possessed relaxing activity in myofibrillar incubation systems, but showed very little ability to accumulate Ca\(^{++}\)-ions. Further analysis, however, showed that the relaxing activity of the material in these zones was probably due to
contamination by a small amount of heavy vesicular material, similar to that isolated from the upper layer of the heavy microsomes. Electron microscopy of the two largest zones from the density gradient-fractionated light microsomes revealed a heterogeneous and poorly characterized composition in these zones. Mitochondrial structures were absent, but further analysis was not attempted. Seraydarian and Mommaerts' study demonstrates clearly that ordinary "crude" microsomal fractions prepared by differential centrifugation are very heterogeneous mixtures. It follows that biochemical activities found in preparations classified as "fragmented relaxing factor" will depend on the mode of preparation of these fractions.

In 1968, Yu et al. described a method for the purification of sarco-tubular membranes from rat skeletal muscle. This method involves fractionation of crude muscle microsomes (isolated between 15,000 - 50,000 x g) by sequential use of two, discontinuous sucrose gradient centrifugation systems, a (35 → 65%) sucrose gradient followed by a (25 → 50%) sucrose gradient. The crude microsomal preparation was resolved into two fractions on the first gradient system. Both of these fractions were able to accumulate Ca^{++} and had Mg^{++}-activated ATPase activity, although the upper fraction was slightly more active in this respect than the lower. The upper fraction from the first gradient centrifugation was further resolved into two subfractions by centrifugation through the second sucrose gradient. Again, both of these subfractions were able to hydrolyze ATP and accumulate Ca^{++} ions although the top subfraction from the second gradient had a higher Mg^{++}-activated ATPase activity and Ca^{++}-uptake activity than the top layer isolated from their
first gradient system. In terms of relative Ca\textsuperscript{++}-binding activity, the upper layer from the second gradient purification would sequester almost twice as much Ca\textsuperscript{++} per mg of protein as the upper layer from the first gradient purification and three to four times as much Ca\textsuperscript{++} as the lower layer from the second gradient purification. Electron micrographs of the two layers from the second gradient purification showed that the upper layer was homogeneous and consisted primarily of membraneous vesicles, whereas the lower layer was not homogeneous and contained many non-vesicular structures as well as some mitochondria. Since these two layers were present as a single layer in the first density gradient centrifugation, Yu's results clearly indicate that the presence of a single band after density gradient centrifugation through discontinuous gradient does not mean that the system is homogeneous.

Density gradient centrifugation was also used by Katz and Repke (1967) to purify crude dog cardiac microsomes. Purified cardiac microsomes, obtained by centrifugation of crude microsomes through either a discontinuous (25 - 60%) or a discontinuous (20 - 35%) sucrose gradient, were able to accumulate 2.23 \(\mu\)mole Ca\textsuperscript{++} per mg of microsomal protein, whereas crude cardiac microsomes before density gradient purification accumulated only 1.9 \(\mu\)mole Ca\textsuperscript{++} per mg microsomal protein.

Schuel et al. (1965) used sucrose zonal centrifugation to obtain relatively "pure" relaxing particles from a crude microsomal fraction isolated from rat muscle between 11,700 x 15 and 73,400 x 60 (g x min). Schuel's microsomes were prepared from skeletal muscles that were homogenized in 0.25 M sucrose, 0.13 M KCl, 20 mM potassium acetate, and
Relaxing factor activity was found only in particles having sedimentation characteristics of microsomes, and not in those particles having sedimentation characteristics of mitochondria. It was also found that even after zonal centrifugation, the microsomal fraction was not completely free of mitochondrial contamination.

Although the density gradient centrifugation procedures just described did result in some improvement in purity of the sarcotubular vesicles, there were several disturbing indications that appreciable heterogeneity still remained in the density-gradient purified microsomes. As was just indicated, Schuel et al. (1965) found that his density-gradient purified preparations were not completely free of mitochondrial contamination as measured by cytochrome oxidase activity. Also, Mommaerts (1967) found evidence for a heterogeneous protein composition in density-gradient purified microsomal preparations that had been stored in sucrose and later washed in phosphate buffer. One common fault of almost all sucrose density gradient purification schemes used up to now for fractionation of muscle microsomes has been their discontinuous nature. This feature could cause difficulty in obtaining "pure" preparations, particularly in view of the heterogeneous and poorly characterized nature of muscle microsomal preparations.

In 1968, Martonosi (1968a) found that sarcotubular vesicle preparations prepared by ordinary differential centrifugation contained an actomyosin-like contaminant. This actomyosin-like contaminant could be found in skeletal muscle microsomes that had been homogenized in 100 mM KCl buffered with 5 mM histidine, pH 7.3, and isolated by differential centrifugation between 8,000 × g for 20 min and 28,000 × g for 60 min.
Extraction of the crude microsomes with 0.6 M KCl removed the actomyosin-like contamination and Martonosi (1968a) suggested that muscle microsomal preparations isolated by differential centrifugation be routinely subjected to a KCl-extraction to remove actomyosin. Uchida et al. (1965) had earlier found myosin in muscle microsomal preparations made by homogenizing rabbit skeletal muscle in 80 mM KCl, 5 mM K₂C₂O₄, and 20 mM histidine, pH 7.2. However, no myosin-like contamination was evident in microsomes that were prepared from muscle homogenized in sucrose media.

The preceding studies plainly show that isolation and purification of fragmented relaxing factor remains a difficult and unsolved problem. It seems probable, however, that application of both a 0.6 M KCl extraction and density gradient purification may provide significant improvement in the homogeneity of sarcotubular vesicle preparations of muscle. This possibility formed the basis for the present study.

Relaxing Factor and Post-Mortem Behavior

The behavior of muscle post-mortem has been of interest to the food scientist as well as the muscle biochemist. To the food scientist, studies with post-mortem muscle have provided some insight into changes in meat quality during the onset and resolution of rigor mortis. To the muscle biochemist, such studies have provided information on the nature of muscle contraction and relaxation, as well as on muscle fine structure. Indeed, as has already been indicated, the relaxing factor system was first discovered during a study of post-mortem muscle. Although a substantial amount of effort has been devoted to the study of the post-mortem behavior of myofibrillar proteins, very little is known about changes in post-mortem
behavior of the sarcoplasmic reticulum. Nauss and Davies (1966) recently used 2, 4-dinitrofluorobenzene to induce rigor in post-mortem muscle strips and indicated that damaged sarcoplasmic reticulum was the probable cause of rigor development in muscle. These investigators simultaneously studied the liberation of \(^{45}\)Ca and the development of rigor tension in \(^{45}\)Ca-labeled muscles which were bathed in Ringer's solution. They found that rate of \(^{45}\)Ca efflux from the treated muscle increased just prior to development of rigor in the muscles.

Further support for the idea that release of Ca\(^{++}\) from the sarcoplasmic reticulum initiates rigor was provided by Kushmerick and Davies (1968) when they studied thaw contraction and development of thaw rigor in frog muscle. Frozen frog muscles, when thawed rapidly, contracted and became stiff and inextensible. This contraction was characterized by a release of Ca\(^{++}\) and rapid utilization of ATP.

Several investigators have reported that preparations of muscle microsomes lose ability to sequester calcium during storage (Ebashi and Lipmann, 1962; Fanburg et al., 1964; Lee et al., 1965; Muscatello et al., 1962; Seraydarian and Mommaerts, 1965; and Yu et al., 1968). However, it is by no means clear that the events in post-mortem sarcoplasmic reticulum can be simulated by simple storage of muscle microsomes in sucrose or KCl solutions. Indeed, in view of the finding that trypsin causes a rapid loss in Ca\(^{++}\) sequestering ability (Ikemoto et al., 1968; Inesi and Asai, 1968; Martonosi, 1968a), it seems likely that lysosomal enzymes may cause a very rapid loss of Ca\(^{++}\)-sequestering ability in post-mortem muscle.

The only study thus far reported which was specifically designed to study
post-mortem events in sarcoplasmic reticulum in situ. Greaser et al. (1967) demonstrated that microsomes isolated from porcine muscle after different times of post-mortem storage had lost much of their ability to sequester calcium. Calcium-accumulating activity of the microsomes (8,000 x 20 - 30,000 x 60 (g x min)) was reduced by 40% during the first three hours of storage and then decreased to 10% of the initial activity after 24 hours post-mortem. However, electron microscopy studies failed to detect any differences in fine structure among preparations made after various post-mortem times. Greaser's microsomal preparations were isolated by simple differential centrifugation, and no evidence was given concerning the purity or composition of these preparations. Thus, some uncertainty exists concerning Greaser's conclusions, particularly since the mitochondrial [2,000 x 20 - 8,000 x 20 (g x min)] and light microsomal [30,000 x 60 - 60,000 x 60 (g x min)] fractions also suffered drastic losses in Ca^{++}-binding activities during post-mortem storage in Greaser's study.

Other reports have indicated that the sarcoplasmic reticulum of cardiac muscle loses functional integrity under abnormal metabolic conditions. Bryant et al. (1958) and Lee et al. (1967) studied the effect of cardiac ischemia on the fine structure and the Ca^{++}-uptake activity of the sarcoplasmic reticulum, respectively. Lee et al. found that the Ca^{++}-sequestering mechanism was impaired in sarcoplasmic reticulum isolated from ischemic cardiac tissue. Bryant and coworkers noted considerable swelling and vesiculation of the sarcoplasmic reticulum in their preparations.
The preceding studies show that Ca$^{++}$-accumulating ability of the sarcoplastic reticulum is a very labile activity which can be affected even by abnormal in vivo conditions. Thus, it may be expected that substantial changes occur in the sarcoplastic reticulum during post-mortem storage. In view of the observations by Mommaerts (1967) and Martonosi (1968a) indicating a heterogeneous protein composition in their microsomal preparations, initial efforts were directed toward improvement of existing techniques for preparation of skeletal muscle microsomes. After development of an improved method for preparation of the fragmented sarcoplastic reticulum, this method was applied to isolation of sarcoplastic reticular fragments from post-mortem muscle in anticipation of learning more about the phenomenon of rigor development.
METHODS AND MATERIALS

This study consisted of two separate but related parts. In the first part, primary effort was directed toward development of a procedure for preparation of purified muscle microsomes. These experiments used principally rabbit muscle although a few experiments for comparative purposes were done using porcine muscle. The second part of the study was initiated only after some experience had been gained toward preparation purified muscle microsomes, and involved investigation of changes in the activity of muscle microsomes in post-mortem muscle. These experiments used rabbit, porcine, and bovine muscle.

Porcine and bovine tissue samples were obtained from animals slaughtered at the Iowa State University Meat Laboratory. Animals were exsanguinated, samples of the longissimus dorsi or semitendinosus muscles were removed as soon as possible after death, and taken at once to the Iowa State University Food Research Laboratory. In studies utilizing rabbits as the source of experimental material, rabbits were first immobilized with 90 mg sodium pentobarbital and 1.5 mg of d-tubocurarine, and then exsanguinated from the carotid arteries. Back and leg muscles were immediately excised, cleaned of fat and connective tissue, and used for subsequent microsomal preparation.

Preparation of all samples was done at $2^\circ\text{C}$ with pre-cooled solutions. All solutions used in this study were prepared with distilled water that was double-deionized and redistilled in glass prior to use. The sucrose and Tris employed for preparation of microsomes was Special Enzyme Grade (Mann Research Laboratories, Inc.). The KCl used was biological grade.
(Allied Chemicals) and all other chemicals were reagent grade. A 0.1 M solution of Hepes-ATP was prepared by passage of sodium ATP (Sigma Chemical Co.) through Dowex-50 in the H⁺ form, followed by neutralization with potassium hydroxide, and addition of the potassium salt of Hepes to 0.3 M final concentration.

Protein concentrations were measured by the biuret method (Cornall et al., 1949), standardized against bovine serum albumin. Standard errors were computed according to the methods outlined by Steele and Torrie (1960).

Preparation of Microsomes

Salient features of the procedures used for preparation of skeletal muscle microsomes are given in Scheme 1. All centrifugation steps were done at 2°C. Muscle was ground in an Oster Model 516 Grinder and then homogenized one minute with five volumes (v/w) of cold 0.25 M sucrose, 1 mM EDTA, 0.05 M Tris, pH 7.6. The homogenization period consisted of three bursts of twenty seconds each, with a forty-second cooling period between bursts.

After centrifugation on the continuous (20 - 60%) sucrose gradient at 106,900 x g for 1½ hr (Scheme 1), the microsomes appeared as two prominent zones in the nitrocellulose centrifuge tubes. These layers were collected by puncturing a small hole in the bottom of the nitrocellulose tubes and collecting the layers in small separate beakers which were held on ice. The collected layers were then transferred to a small glass homogenizer where they were gently homogenized with five strokes of a Teflon pestle. The homogenates were then stored on ice for subsequent analysis.
Scheme 1. Flowsheet for fractionation of skeletal muscle homogenates and preparation of microsomes
Procedure for the Preparation of Skeletal Muscle Microsomes

**Ground muscle tissue**

1) Homogenize in 5 volumes of 0.25 M sucrose, 0.05 M Tris, 1 mM EDTA, pH 7.6 for 60 sec
2) Centrifuge at 2,600 x g for 15 min

**Sediment**  
1) Homogenize in 5 volumes of 0.25 M sucrose, 0.05 M Tris, 1 mM EDTA, pH 7.6 for 10 sec  
2) Centrifuge at 2,600 x g for 15 min

**Supernatant**

1) Centrifuge at 53,679 x g for 2 hr

**Combined supernatants**

1) Strain through 4 layers of cheesecloth to remove lipid material
2) Centrifuge at 8,200 x g for 30 min

**Sediment**  
1) Suspend in 0.25 M sucrose by 5 strokes of a Teflon pestle in a glass homogenizer

**Supernatant**

1) Layer 3-4 ml on 55 ml of a continuous (20 - 60%) sucrose gradient
2) Centrifuge at 106,900 x g for 1 1/2 hr

**Sediment** — crude microsomes
1) Remove from gradient and pellet at 105,651 x g for 1 hr

Supernatant (Discard)

Sediment — suspend in 0.25 M sucrose by five strokes of a Teflon pestle in a glass homogenizer

Supernatant (Discard)

Sediment — Gradient-purified microsomes — suspend in 0.25 M sucrose by five strokes of a Teflon pestle in a glass homogenizer

Scheme 1 (Continued)
Some preliminary studies showed that the ATPase and Ca$^{++}$-uptake activities of even these fractions were quite variable, so an additional fractionation step involving extraction with 0.6 M KCl was added. The protein extracted from the microsomes by 0.6 M KCl was precipitated by dilution, dissolved in 1.0 M KCl and its sedimentation behavior examined in the analytical ultracentrifuge. Also, the ATPase activity of this protein was measured by a slight modification of the procedure of Goll and Robson (1967). The procedure for the KCl extraction is shown in Scheme 2. In some instances, the KCl-extracted microsomes (Scheme 2) were further extracted by acetone. The acetone extraction procedure is also shown in Scheme 2.

An attempt was made to further purify the density-gradient purified microsomes (Scheme 1) by subjecting them to a second density gradient purification as shown in Scheme 3. This second density-gradient step also resulted in the appearance of two zones which were collected as described previously. Samples for ATPase activity and Ca$^{++}$-accumulation studies were taken before the first density gradient centrifugation, from the top layer resulting from the first density gradient centrifugation, from the top layer resulting from the second density gradient centrifugation, and after KCl extraction of microsomes obtained from the top layer of the second density gradient. The results indicated that extraction of the microsomes with 0.6 M KCl produced the greatest increase in both ATPase activity and calcium sequesteration. Therefore, microsomes prepared by differential centrifugation [$8,200 \times 30 \rightarrow 53,679 \times 120$ (g x min)] were subjected directly to extraction with 0.6 M KCl before any density gradient purification. The KCl-extracted crude microsomes were then subjected to
Scheme 2. KCl and acetone extraction of gradient-purified muscle microsomes
KCl and Acetone Extraction of Gradient-Purified Muscle Microsomes

**Pellet of gradient — purified microsomes**

1) Homogenize in 0.6 M KCl with five strokes of a Teflon pestle in a glass homogenizer
2) Centrifuge at 105,651 x g for 30 min

**Supernatant 1**

1) Dilute tenfold using double deionized distilled H₂O at 2°C
2) Centrifuge at 11,900 x g for 40 min

**Combined supernatants 1 and 2**

1) Dissolve in 1.0 M KCl
2) Clarify at 20,000 x g for 10 min

**Sediment**

1) Homogenize in 0.6 M KCl with five strokes of a Teflon pestle in a glass homogenizer
2) Centrifuge at 105,651 x g for 30 min

**Sediment — KCl-extracted gradient-purified microsomes**

**Supernatant 2**

**Sediment (Discard)**

**Supernatant (Discard)**

**Supernatant — myosin solution**
1) Suspend in one of the following medium with a glass homogenizer equipped with a Teflon pestle
   a) Dry acetone
   b) 5% water in acetone (v/v)
   c) 10% water in acetone (v/v)

2) Centrifuge at 1,200 x g for 10 min

Supernatant (Discard)  Sediment — Acetone extracted microsomes - suspend in 0.25 sucrose by five strokes of a Teflon pestle in a glass homogenizer

Scheme 2 (Continued)
Scheme 3. Flowsheet for fractionation of gradient-purified muscle microsomes
Fractionation of Gradient-Purified Muscle Microsomes

Gradient-purified microsomes (Scheme 1)
1) Layer 3-4 ml on 55 ml of a continuous (20 - 60%) sucrose gradient
2) Centrifuge at 106,900 x g for 1 1/2 hr

Bottom layer
(Discard)

Top layer
1) Suspend in 0.25 M sucrose with a glass homogenizer equipped with a Teflon pestle
2) Centrifuge at 105,651 x g for 1 hr

Supernatant
(Discard)

Sediment — Microsomes were either suspended in 0.25 M sucrose with a glass homogenizer equipped with a Teflon pestle and used directly or were extracted twice with 0.6 M KCl as shown in Scheme 2 to make KCl-extracted microsomes
density gradient purification and the ATPase and Ca\(^{++}\)-sequestering activities of the density gradient purified, KCl-extracted, microsomes were compared to the ATPase and Ca\(^{++}\)-sequestering activities of density gradient purified microsomes which were not subjected to KCl extraction (Scheme 4). Also, as shown in Scheme 4, a few experiments were done in which fractionation on a continuous (20 → 60%) sucrose gradient containing a uniformly distributed concentration of 0.15 M KCl was compared to fractionation on the more generally used continuous (20 → 60%) sucrose gradient which did not contain any KCl. These experiments were also done both with KCl-extracted microsomes and with crude microsomes not treated with KCl (the latter is not shown in Scheme 4).

Post-Mortem Studies

Two types of experiments involving post-mortem muscle were done in this study. In the first type of experiment, the effect of storage at either 2\(^{\circ}\) or 23\(^{\circ}\)C for 24 hr post-mortem was investigated. Muscle samples from the longissimus dorsi of four pigs and four rabbits were obtained as soon as possible after exsanguination. After being wrapped in three layers of Saran Wrap, portions of these samples were stored for 24 hrs at either 2\(^{\circ}\)C or 23\(^{\circ}\)C while the rest of the muscle was used for microsome preparation immediately after death. Crude microsomes were prepared by differential centrifugation as described in Scheme 1. The crude microsomes were then extracted with 0.6 M KCl as shown in Scheme 4 and the KCl-extracted crude microsomes were used directly in the subsequent experiments without undergoing any density gradient fractionation.
Scheme 4. Flowsheet for fractionation of crude muscle microsomes
**KCl-Extraction of Crude Muscle Microsomes**

**Pellet of crude microsomes (Scheme 1)**

1. Homogenize in 0.6 M KCl with 5 strokes of a Teflon pestle in a glass homogenizer
2. Centrifuge at 105,651 x g for 1 hr

**Sediment**

1. Homogenize in 0.6 M KCl with 5 strokes of a Teflon pestle in a glass homogenizer
2. Centrifuge at 105,651 x g for 1 hr

**Suspension of KCl-extracted crude microsomes**

1. Layer 3-4 ml on 55 ml of a continuous (20 → 60%) sucrose gradient
2. Centrifuge at 106,900 x g for 1 1/2 hr

**Suspensions of KCl-extracted crude microsomes**

1. Layer 3-4 ml on 55 ml of a continuous (20 → 60%) sucrose gradient containing 0.15 M KCl
2. Centrifuge at 106,900 x g for 1 1/2 hr

**Top layer**

1. Remove from gradient and pellet at 105,651 x g for 1 hr

**Bottom layer**

1. Remove from gradient and pellet at 105,651 x g for 1 hr

1. Remove from gradient and pellet at 105,651 x g for 1 hr

1. Remove from gradient and pellet at 105,651 x g for 1 hr
Sediment —
suspend in 0.25 M sucrose with a glass homogenizer equipped with a Teflon pestle

Scheme 4 (Continued)
The second type of post-mortem experiment attempted to relate loss of the ability of microsomes to accumulate calcium to the development of post-mortem tension in muscle strips. Psoas major muscle from two rabbits and semitendinosus muscle from one bovine animal were used in this study. Samples were taken immediately after death and muscle strips were placed on an E and M Physiograph (E & M Instrument Co., Inc., Houston, Texas 77077). The strips were bathed in a solution containing 0.08 M KCl, 0.06 M Tris (pH 7.2), 1 mM sodium azide; and 5 mM MgCl₂. In the study involving rabbits, two strips were removed from the physiograph at each of the following times: 1) at the onset of tension development, 2) at the peak of tension development. In the study involving bovine muscle only one strip was taken at each time since the effect of two temperatures on tension development was included in the study. The rabbit muscle strips were held in a temperature controlled incubator that was maintained at 37°C during the course of the experiment whereas the bovine muscle strips were maintained at either 2°C or 37°C during the course of the experiment.

KCl extracted-crude microsomal fractions were prepared from these strips as described for the first type of post-mortem experiment, except that the microsomes were extracted only once with 0.6 M KCl.

ATPase and Ca²⁺ Uptake

Calcium uptake and ATPase activity of the microsomes were measured in an assay system containing 0.1 M KCl, 30 mM Hepes (pH 7.2), 5 mM ATP, 5 mM MgCl₂, 5 mM K₂C₂O₄, 0.15 mM ⁴⁵CaCl₂, and 0.05 mg microsomal protein/ml. The total volume of the incubation medium was 5 ml, and the assay was run at 25°C. The reaction was started by addition of microsomes, and
continued for 15 minutes, after which a two-ml aliquot of the reaction mixture was added to 0.5 milliliter of 15% TCA. The remaining three ml of reaction mixture was filtered through a 0.45μ type HA Millipore filter (Martonosi and Feretos, 1964a). The two ml of reaction mixture which was added to TCA, was analyzed for inorganic phosphate by the method of Taussky and Shorr (1953). A mixture for liquid scintillation was made by adding 1.5 g of Cab-o-sil (Packard) to a 1:1 (v/v) toluene-ethanol solution containing 0.3 g of dissolved PPO. One-half ml aliquots of the Millipore filtrate were added to this mixture and counts of 45Ca-radioactivity were measured in a Packard liquid scintillation counter. A Vortex mixer (Adams) was used to facilitate distribution of calcium through the scintillation gel.

In some studies 5 mM sodium azide was included in the reaction mixture to test for presence of mitochondrial contamination in the microsomal fraction. Mitochondrial ATPase is inhibited by azide (Brierley et al., 1964; Schwartz and Laseter, 1963; Siekevitz et al., 1958), but microsomal ATPase is not affected by this chemical.

**Ultracentrifuge Assays**

Ultracentrifuge assays were done at 20°C using a Spinco Model E analytical ultracentrifuge equipped with a schlieren optical system. Sedimentation coefficients were measured with a Nikon comparator and were calculated in Svedberg units.

**Specimen Preparation for Electron Microscopy**

Microsomal fractions isolated by differential or density gradient centrifugation and then suspended in 0.25 M sucrose were pelleted at
105,651 x g for 1 hr. The pellets were fixed in cold 2.5% glutaraldehyde containing 0.2 M Sorenson's phosphate buffer, pH 7.2. After one hour of fixation, the glutaraldehyde solution was drawn off and replaced with fresh fixative. At the end of the second hour, the glutaraldehyde was removed, and the fixed pellet was washed with Sorenson's phosphate buffer for approximately one to two hours. The pellets were carefully removed from the centrifuge tubes by use of a spatula, placed on polyethylene strips and covered with 0.2 M phosphate buffer. They were then cut into small cubes, and finally post-fixed with 1% osmium tetroxide (buffered with Sorenson's buffer) for two hours. Subsequent tissue dehydration was accomplished through a graded series of acetone, and was followed by infiltration and embedding in an Araldite-Epon mixture. Polymerization of the embedding media was allowed to continued for 24 hours at 60°C. The specimen blocks were sectioned with glass knives on a LKB ultramicrotome. Sections with gray to silver interference colors were placed on uncoated, 300 mesh, copper grids, stained 25 minutes with 2% uranyl acetate in methanol and rinsed in two changes each of methanol, 50% methanol, and glass distilled water. Sectioned materials were examined with an RCA EMU-4 electron microscope operated at an accelerating voltage of 50 kv.
RESULTS

In view of the results suggesting that skeletal muscle microsomes possess a heterogeneous protein composition (Mommaerts, 1967; Martonosi, 1968a) initial efforts in this study were directed toward development of a method for isolation of relatively pure skeletal microsomes. These efforts involved the use of differential centrifugation, high ionic-strength KCl extraction, and conventional small-scale density gradient techniques. After acquiring the experience in preparation of muscle microsomes necessary to permit selection of the best combination of techniques for isolation of purified microsomes, a second study was conducted to gain some insight into possible functional and structural changes in the microsomal fraction during post-mortem storage of muscle.

Purification of Skeletal Muscle Microsomes

The ability of various microsomal fractions isolated from rabbit skeletal muscle to accumulate Ca\(^{++}\) and hydrolyze ATP is compared in Table 1. Centrifugation of crude microsomes through a continuous (20 - 60%) sucrose density gradient resulted in formation of two major zones; these are designated as the top and bottom layers (cf. Figure 7, part a). Both the top and bottom layers sequester calcium and have an active ATPase, although the upper layer is more active in both these respects. Extraction of the upper microsomal layer with 0.6 M KCl produced a 50% increase in the ATPase activity and a 25% increase in Ca\(^{++}\)-sequestering ability of the gradient-purified microsomes. Further extraction of the KCl fraction with either dry acetone, five percent water in acetone (v/v),
Table 1. Ca\textsuperscript{++} sequestration and ATPase activity in rabbit skeletal muscle microsomes after various treatments\textsuperscript{a}

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ATPase activity (\mu\text{moles P}_4/\text{mg protein/min} ) [Mean (6) ± S.E.]</th>
<th>Ca\textsuperscript{++} sequestration (\mu\text{moles Ca}^{++}/\text{mg protein} ) [Mean (6) ± S.E.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Top layer, gradient</td>
<td>0.412 ± 0.005</td>
<td>1.97 ± 0.10</td>
</tr>
<tr>
<td>2. Bottom layer, gradient</td>
<td>0.351 ± 0.006</td>
<td>1.18 ± 0.12</td>
</tr>
<tr>
<td>3. Top layer, gradient, extracted twice with 0.6 M KCl</td>
<td>0.639 ± 0.011</td>
<td>2.44 ± 0.02</td>
</tr>
<tr>
<td>4. KCl-extracted top layer from gradient, extracted with dry acetone</td>
<td>0.302 ± 0.019</td>
<td>0.92 ± 0.10</td>
</tr>
<tr>
<td>5. KCl-extracted top layer from gradient, extracted with 5% H\textsubscript{2}O in acetone (v/v)</td>
<td>0.166 ± 0.043</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>6. KCl-extracted top layer from gradient, extracted with 10% H\textsubscript{2}O in acetone (v/v)</td>
<td>0.189 ± 0.011</td>
<td>0.16 ± 0.05</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Conditions of assay: 0.1 M KCl, 30 mM Hepes (pH 7.2), 0.05 mg protein/ml, 5 mM ATP, 5 mM MgCl\textsubscript{2}, 5 mM K\textsubscript{2}C\textsubscript{2}O\textsubscript{4}, 0.15 mM \textsuperscript{45}CaCl\textsubscript{2}; total volume 5 ml; 25°C. Assays were run for 15 minutes.

or ten percent water in acetone (v/v) leads to a 50% or more decrease in ATPase and Ca\textsuperscript{++}-sequestering activities.

The results obtained from the gradient centrifugation are similar to those reported by Seraydarian and Mommaerts (1965) who observed two major subfractions after centrifugation of a very similar fraction (microsomes isolated by differential centrifugation between 15,000 x g for 20 min and 56,593 x g for 90 min) through a discontinuous (35 – 70%) sucrose gradient. Seraydarian and Mommaerts also found Ca\textsuperscript{++}-sequestering ability
and ATPase activity in both the upper and lower layers isolated from their heavy microsomal fraction. However, the results from their Ca^{++}-uptake studies were quite variable and neither the upper or lower layer exhibited any clear advantage in calcium-accumulating ability. The ATPase activities of both fractions were also quite similar. Table 1 is in agreement with the work of Martonosi (1964; Martonosi et al., 1968), who found that extraction of microsomes with acetone containing ten percent water causes almost complete removal of phospholipids from the membranes and inhibits the Ca^{++}-transport and ATPase activities of the microsomes.

A substantial amount of protein was removed by 0.6 M KCl extraction of the upper layer of density-gradient purified microsomes. Since removal of this protein increased the ATPase and Ca^{++}-sequestering activities of the microsomes, several experiments were done in an effort to elucidate the nature of the extracted protein. Inspection of Table 2 reveals that

Table 2. ATPase activity of protein extracted from muscle microsomes by 0.6 M KCl^a

<table>
<thead>
<tr>
<th>Activator</th>
<th>ATPase activity μmoles P_i/mg protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg^{++}</td>
<td>.037 ± .003b</td>
</tr>
<tr>
<td>Ca^{++}</td>
<td>.082 ± .002</td>
</tr>
</tbody>
</table>

^aConditions of assay: 50 mM KCl, 30 mM Hepes, (pH 7.2), 1 mM MgCl₂ or CaCl₂, 1 mM ATP, 0.2 mg protein/ml; volume, 2.5 ml; 25°C. Assay stopped with 1/2 ml TCA after 15 minutes.

^bValues are expressed as means plus or minus standard errors. Figures are based on six determinations.
the KCl-extracted protein contains an ATPase activity which, in the presence of 50 mM KCl at pH 7.2, is activated by 1 mM Ca^{++} and slightly inhibited by 1 mM Mg^{++}. Examination of two of the KCl-extracts in the analytical ultracentrifuge showed that most of the protein in these extracts sedimented as a single sharp boundary with S values of 4.80 and 5.45 when the protein concentration of the fractions were 6.13 and 2.44 mg/ml, respectively (Figure 1). A small amount of faster sedimenting material (S = 5.55 at 6.13 mg/ml and 7.07 at 2.44 mg/ml) was also evident in the sedimentation diagrams (Figure 1).

Results of the analytical ultracentrifugation studies and the ATPase data suggest that the protein extracted from microsomes by 0.6 M KCl is myosin. Myosin exhibits a very concentration-dependent sedimentation behavior with an S value near 6.0-6.2 when extrapolated to infinite dilution, and also has a pronounced aggregating tendency (Lowey and Cohen, 1962). This aggregating tendency may account for the small leading peak in the sedimentation diagrams in Figure 1. Furthermore, myosin has a Ca^{++}-activated ATPase activity and is slightly inhibited by Mg^{++} at 50 mM KCl and pH 7.2. The finding that myosin is present in crude microsomes isolated in sucrose is not in agreement with the results of Uchida et al. (1965) who reported that homogenization of muscle in sucrose-containing media prevented myosin contamination of muscle microsomes isolated between 10,000 x g and 40,000 x g for one hr.

The results described in Table 1 indicate that a single density-gradient purification, even on a continuous sucrose gradient, did not result in completely purified skeletal muscle microsomes free from contaminants.
Figure 1. Sedimentation pattern of protein extracted by 0.6 M KCl from material in the top layer of a continuous (20 → 60%) sucrose gradient purification of muscle microsomes. Solvent: 1.0 M KCl. Concentration of protein: 6.13 mg/ml. The run was made on a Spinco model E analytical ultracentrifuge at 20°C.
MINUTES AFTER REACHING SPEED OF 59,780 RPM

0.6M KCl EXTRACT OF MICROSONES
such as myosin. Therefore, the procedure for purification of microsomes was extended by layering the top layer collected from the first gradient onto a second continuous (20–60%) sucrose gradient. The microsomal subfraction layered on this second gradient also separated into two zones which were located near the top and near the center of the (20–60%) sucrose gradient after centrifugation at 25,000 rpm for 90 min.

Table 3 shows results comparing the Ca$^{++}$-sequestration and ATPase activities of (a) crude microsomes, (b) microsomes from the top layer of first density gradient centrifugation, (c) microsomes from the top layer of the second density gradient centrifugation, and (d) microsomes from the top layer of second density gradient centrifugation which have subsequently been extracted with 0.6 M KCl. The results indicate that a second density-gradient purification of density-gradient-purified microsomes from rabbit skeletal muscle causes only a slight improvement in either Ca$^{++}$-uptake and ATPase activities of the microsomes. It is obvious, however, that the first density gradient purification does produce a significant purification, in terms of either Ca$^{++}$-uptake or ATPase activity, over crude microsomes prepared by simple differential centrifugation. Extraction of the upper layer from the second density gradient centrifugation with 0.6 M KCl results in a 15–40% increase in ATPase activity and Ca$^{++}$-accumulating capacity of the microsomes. This improvement in specific activity occurs even though the microsomes have been subjected to two purification cycles through sucrose density gradients. Electron microscopic examination of the microsomal fractions isolated by simple differential centrifugation showed that the top part of the
Table 3. Ca$^{++}$ sequestration and ATPase activity in crude and purified fractions of rabbit skeletal muscle microsomes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ATPase activity $\mu$moles P$_i$/mg protein/min [Mean (6) ± S.E.]</th>
<th>Ca$^{++}$ sequestration $\mu$moles Ca$^{++}$/mg protein [Mean (6) ± S.E.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 8,200 - 53,679 x $g$</td>
<td>.353 ± .026</td>
<td>1.50 ± 0.10</td>
</tr>
<tr>
<td>2. Top layer, gradient 1</td>
<td>.422 ± .007</td>
<td>1.92 ± 0.04</td>
</tr>
<tr>
<td>3. Top layer, gradient 2</td>
<td>.476 ± .012</td>
<td>2.08 ± 0.02</td>
</tr>
<tr>
<td>4. Top layer, gradient 2</td>
<td>.654 ± .007</td>
<td>2.37 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>extracted twice with</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6 M KCl</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Conditions of assay: 0.1 M KCl, 30 mM Hepes (pH 7.2), 0.05 mg protein/ml, 5 mM ATP, 5 mM MgCl$_2$, 5 mM K$_2$C$_2$O$_4$, 0.15 mM $^{45}$CaCl$_2$; total volume 5 ml; 25°C. Assays were run for 15 min.

crude microsomal pellet (the 8,200 - 53,679 x $g$ fraction) consisted almost entirely of vesicular material, whereas the extreme bottom of the pellet contained mostly vesicular material which had some slight mitochondrial contamination (Figure 2a and 2b).

During the progress of this study, Yu et al. (1968) published a method for fractionation of microsomes isolated from rat skeletal muscle. This study used purification through two successive sucrose density gradients and was very similar to the sequential sucrose gradient purification just described in the present study. However, Yu et al. used discontinuous gradients and found that two sequential sucrose density gradients steps caused about a 40% increase in Ca$^{++}$-uptake and ATPase activity of their microsomal fractions, when compared to microsomes isolated from a single density gradient purification.
Figure 2. Electron micrograph of rabbit skeletal microsomes isolated by differential centrifugation between 8,200 x g for 30 min and 53,679 x g for 2 hr. Pellets from the differential centrifugation were fixed in glutaraldehyde and postfixed with osmium tetroxide. Sections were stained with methanolic uranyl acetate.


2b. Bottom of pellet from 8,200 - 53,679 x g fraction. Note vesicular (V) material and mitochondria (M). Magnification: X 29,000.
The experiments described in Tables 1 and 3 show that extraction by 0.6 M KCl causes the greatest increase in ATPase and \( \text{Ca}^{++} \)-sequestration activities of muscle microsomes, even after the microsomes had been purified through two successive sucrose density gradients. Therefore, an experiment was done in which the crude microsomal pellet, isolated by differential centrifugation, was immediately subjected to 0.6 M KCl extraction to determine whether direct extraction of crude microsomes would also result in significant purification. Animals from two different species, rabbit and porcine, were used in this experiment, and the results are summarized in Tables 4 and 5. Extraction of the crude microsomes with 0.6 M KCl resulted in almost a 100% increase in microsomal ATPase activity and a 30-35% increase in microsomal \( \text{Ca}^{++} \)-sequestering ability. Comparison of activities in Tables 1, 3, and 4 show that direct KCl extraction of crude microsomes results in \( \text{Ca}^{++} \)-sequestering and ATPase activities as high as those produced by KCl extraction of density-gradient purified microsomes. Thus, it appears that direct extraction by a high ionic strength solution is a very effective, single treatment for purification of crude microsomes.

The results shown in Tables 4 and 5 also indicate that microsomal ATPase is insensitive to 5 mM sodium azide. It has been shown (Brierley et al., 1964; Fanburg and Gergely, 1965; Schwartz and Laseter, 1963) that the ATPase activity of crude microsomes is inhibited only slightly by azide which is a potent inhibitor of both the ATPase activity and calcium uptake of mitochondria. The failure of azide to decrease calcium accumulation by muscle microsomes is supported by the findings of Katz and
Table 4. Effect of azide and extraction by 0.6 M KCl on the ATPase activity and Ca\(^{++}\) sequestration activities of rabbit skeletal muscle microsomes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ATPase activity (\mu\text{moles }\text{P}_i/\text{mg protein/min}) [Mean (6) ± S.E.]</th>
<th>Ca(^{++}) sequestration (\mu\text{moles Ca}^{++}/\text{mg protein}) [Mean (6) ± S.E.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium azide</td>
<td>.341 ± .014</td>
<td>1.70 ± .06</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>.333 ± .008</td>
<td>1.60 ± .10</td>
</tr>
</tbody>
</table>

1. \(8,200 - 53,679 \times g\)

---

Table 5. Effect of sodium azide and extraction by 0.6 M KCl on the ATPase activity and Ca\(^{++}\) sequestration activities of microsomes from porcine longissimus dorsi muscle

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ATPase activity (\mu\text{moles }\text{P}_i/\text{mg protein/min}) [Mean (10) ± S.E.]</th>
<th>Ca(^{++}) sequestration (\mu\text{moles Ca}^{++}/\text{mg protein}) [Mean (10) ± S.E.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium azide</td>
<td>.356 ± .016</td>
<td>1.59 ± .04</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>.361 ± .017</td>
<td>1.52 ± .07</td>
</tr>
</tbody>
</table>

1. \(8,200 - 53,679 \times g\)

---

\(^a\)Conditions of assay: 0.1 M KCl, 30 mM Hepes (pH 7.2), 0.05 mg protein/ml, 5 mM ATP, 5 mM MgCl\(_2\), 5 mM K\(_2\)C\(_2\)O\(_4\), 0.15 mM \(^{45}\text{CaCl}_2\); total volume, 5 ml; 25°C. Assays were run for 15 min.

\(^b\)In assays containing sodium azide, concentration was 5 mM.

---

\(^a\)Conditions of assay: 0.1 M KCl, 30 mM Hepes (pH 7.2), 0.05 mg protein/ml, 5 mM ATP, 5 mM MgCl\(_2\), 5 mM K\(_2\)C\(_2\)O\(_4\), 0.15 mM \(^{45}\text{CaCl}_2\); total volume, 5 ml; 25°C. Assays were run for 15 min.

\(^b\)In assays containing sodium azide, concentration was 5 mM.
Repke (1967) who found that calcium uptake by cardiac microsomes is only slightly inhibited by sodium azide. Thus, although it cannot be established that \( \text{Ca}^{++} \)-accumulating microsomal fractions prepared by differential centrifugation are derived exclusively from sarcoplasmic reticulum, it is at least highly unlikely that mitochondrial contamination contributes significantly to the calcium uptake observed for the microsomal fractions.

The results in Table 5 show that microsomes from porcine *longissiumus dorsi* behave very similarly to those isolated from rabbit muscle. Again, immediate extraction of crude microsomes by 0.6 M KCl produces a significant increase in both the ATPase and \( \text{Ca}^{++} \)-sequestering activities. These activities in purified porcine muscle microsomes are almost identical to the corresponding activities in purified rabbit muscle microsomes (cf. Table 4).

Electron microscopic observations on pellets of crude microsomes and KCl-extracted crude microsomes are shown in Figures 3-6. Although the KCl-extracted rabbit microsomes shown in Figure 4 appear to contain a higher proportion of flattened vesicles, this difference was not generally observable throughout the pellet. KCl-extracted microsomes did consistently appear to contain much less electron opaque material than crude microsomes. Mitochondrial fragments, recognizable by their cristae, were observed in only a very few of these preparations.

The preceding experiments showed that immediate extraction of crude microsomes with 0.6 M KCl produces the same degree of purity, as measured by \( \text{Ca}^{++} \)-sequestering and ATPase activities, as did two successive density
Figure 3. Electron micrograph of rabbit skeletal muscle microsomes. The microsomal fraction was sedimented between $8,200 \times g$ for 30 min and $53,679 \times g$ for 120 min. Pellets from the differential centrifugation were fixed in glutaraldehyde and postfixed with osmium tetroxide. Section was stained with methanolic uranyl acetate. Magnification: $X \ 61,800$.

Figure 4. Electron micrograph of rabbit skeletal microsomes. The microsomal was sedimented between $8,200 \times g$ for 30 min and $53,679 \times g$ for 120 min, then extracted twice with 0.6 M KCl, and the extracted microsomes sedimented at $105,651 \times g$ for 60 min. The resulting pellet was fixed in glutaraldehyde and postfixed with osmium tetroxide. Section was stained with methanolic uranyl acetate. Magnification: $X \ 43,700$. 
Figure 5. Electron micrograph of microsomes from porcine *longissimus dorsi*. The microsomal fraction was sedimented between 8,200 x g for 30 min and 53,679 x g for 120 min. Pellets from the differential centrifugation were fixed in glutaraldehyde and postfixed with osmium tetroxide. Section was stained with methanolic uranyl acetate. Magnification: X 79,800.

Figure 6. Electron micrograph of microsomes from porcine *longissimus dorsi*. The microsomal fraction was sedimented between 8,200 x g for 30 min and 53,679 x g for 120 min, then extracted twice with 0.6 M KCl and the extracted microsomes sedimented at 105,651 x g for 60 min. The resulting pellet was fixed in glutaraldehyde and postfixed with osmium tetroxide. Section was stained with methanolic uranyl acetate. Magnification: X 79,800.
gradient purifications followed by 0.6 M KCl extraction. Thus, under the conditions used in this study, a 0.6 M KCl extraction appears to be the most effective purification technique that can be applied to crude microsomes.

A fourth experiment was done to determine the effectiveness of density gradient purification when done following instead of preceding the 0.6 M KCl extraction. This experiment was designed to afford direct comparison of (a) crude microsomes isolated in sucrose media, (b) crude microsomes isolated in sucrose followed by 0.6 M KCl extraction, (c) crude microsomes layered on either a continuous (20 → 60%) sucrose gradient or on a continuous (20 → 60%) sucrose gradient containing a uniformly distributed concentration of 0.15 M KCl, and (d) crude microsomes, extracted with 0.6 M KCl, and then layered on one of a set of gradients similar to those described in (c). When the crude or KCl-extracted microsomes were fractionated on continuous (20 → 60%) sucrose density gradients, or on such gradients containing a uniform concentration of 0.15 KCl, two layers appeared. The positions of these layers after centrifugation at 25,000 rpm for 90 min in the gradient systems employed are diagrammatically shown in Figure 7. Neither inclusion of 0.15 M KCl in the sucrose gradient nor prior extraction of the microsomes with 0.6 M KCl caused any large change in distribution of the microsomal subfractions on sucrose gradients. Usually, however, the layers in the sucrose gradients containing KCl did not appear as clearly defined and sharp as those in pure sucrose gradients. It was also noticed that prior extraction with 0.6 M KCl appeared to cause slightly narrower zones upon density gradient centrifugation.
Figure 7. Diagrams showing the distribution of microsomal subfractions in sucrose density gradients.

7a. Distribution of subfractions of microsomes isolated in sucrose and centrifuged directly in a (20 - 60%) sucrose gradient.

7b. Distribution of subfractions of microsomes after centrifugation in a (20 - 60%) sucrose gradient containing a linear concentration of 0.15 M KCl.

7c. Distribution of subfractions of microsomes isolated in sucrose, extracted twice with 0.6 M KCl and then centrifuged in a (20 - 60%) sucrose gradient.

7d. Distribution of subfractions of microsomes isolated in sucrose, extracted twice with 0.6 M KCl and centrifuged in a (20 - 60%) sucrose gradient containing a linear concentration of 0.15 M KCl.
Yields of particulate material isolated by differential centrifugation and after purification in the different gradient systems just described are presented in Table 6. Extraction with 0.6 M KCl causes the loss of approximately 30% of the protein in the crude microsomes. However, regardless of whether the crude microsomes had been extracted with KCl, over 40% of the protein in the crude microsomal fraction appeared in the upper layer following density gradient centrifugation. There is some indication that less of the crude microsomal protein was recovered in the two principal layers when 0.15 M KCl was included in the gradient. Most of this missing material was apparently lost from the upper layer. About 70% of the crude microsomal protein was recovered in the two layers from sucrose density gradients and about 60% was recovered in the two layers when 0.15 M KCl was included in the sucrose gradient. Moreover, even after extraction with 0.6 M KCl, about 70% of the KCl-extracted protein was recovered in the two layers from sucrose density gradient and about 60% was recovered in the two layers when 0.15 M KCl was included in the sucrose gradient. Thus, removal of myosin impurities by KCl-extraction does not appear to affect recovery of microsomal protein from sucrose density gradients. The yields reported in Table 6 correspond closely to those reported by Seraydarian and Mommaerts (1965) for density gradient purification of muscle microsomes.

The Ca\(^{++}\) sequestering and ATPase activities of the various microsomal subfractions before and after density gradient centrifugation are presented in Table 7. These data confirm the previous results in this study that the Ca\(^{++}\)-accumulating capacity and ATPase activity of crude
Table 6. Yields of particulate material after isolation of various fractions of rabbit microsomes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Gradient</th>
<th>mg protein/gm muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 8,200 - 53,679 x g</td>
<td>(20 → 60% sucrose)</td>
<td>3.37</td>
</tr>
<tr>
<td>Top layer</td>
<td>1.74</td>
<td></td>
</tr>
<tr>
<td>Bottom layer</td>
<td>.46</td>
<td></td>
</tr>
<tr>
<td>(20 → 60% sucrose 0.15 M KCl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top layer</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>Bottom layer</td>
<td>.51</td>
<td></td>
</tr>
<tr>
<td>2. 8,200 - 53,679 x g</td>
<td>extracted twice with 0.6 M KCl</td>
<td>2.40</td>
</tr>
<tr>
<td>(20 → 60% sucrose)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top layer</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>Bottom layer</td>
<td>.46</td>
<td></td>
</tr>
<tr>
<td>(20 → 60% sucrose 0.15 M KCl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top layer</td>
<td>.97</td>
<td></td>
</tr>
<tr>
<td>Bottom layer</td>
<td>.46</td>
<td></td>
</tr>
</tbody>
</table>
Table 7. Ca\textsuperscript{++} sequestration and ATPase activity of crude and purified rabbit skeletal muscle microsomes\textsuperscript{a}

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Gradient</th>
<th>ATPase activity</th>
<th>Ca\textsuperscript{++} sequestration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μmoles P\textsubscript{i}/mg protein/min [Mean (12) ± S.E.]</td>
<td>μmoles Ca\textsuperscript{++}/mg protein [Mean (12) ± S.E.]</td>
</tr>
<tr>
<td>1. 8,200 - 53,679 x g</td>
<td>(20 → 60% sucrose) Top layer</td>
<td>.527 ± .021</td>
<td>2.24 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bottom layer</td>
<td>.424 ± .011</td>
</tr>
<tr>
<td></td>
<td>(20 → 60% sucrose, 0.15 M KCl) Top layer</td>
<td>.535 ± .018</td>
<td>2.39 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bottom layer</td>
<td>.413 ± .013</td>
</tr>
<tr>
<td>2. 8,200 - 53,679 x g fraction extracted twice with 0.6 M KCl</td>
<td>(20 → 60% sucrose) Top layer</td>
<td>.609 ± .007</td>
<td>2.44 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bottom layer</td>
<td>.592 ± .011</td>
</tr>
<tr>
<td></td>
<td>(20 → 60% sucrose, 0.15 M KCl) Top layer</td>
<td>.634 ± .007</td>
<td>2.53 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bottom layer</td>
<td>.600 ± .009</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Conditions of assay: 0.1 M KCl, 30 mM Hepes (pH 7.2), 0.05 mg protein/ml, 5 mM ATP, 5 mM MgCl\textsubscript{2}, 5 mM K\textsubscript{2}C\textsubscript{2}O\textsubscript{4}, 0.15 mM \textsuperscript{45}CaCl\textsubscript{2}; total volume, 5 ml; 25°C. Assays were run for 15 minutes.

Microsomes is greatly improved by simple extraction of the crude microsomes with 0.6 M KCl. Martonosi (1968a) has found that a high ionic strength extraction removed a myofibrillar contaminant from his microsomal preparation, but he did not show any data comparing the ATPase and Ca\textsuperscript{++}-sequestering activities of his preparation before and after KCl-extraction.
It is obvious from Table 7 that KCl-extraction of crude microsomes results in a larger improvement in microsomal ATPase and Ca\textsuperscript{++}-sequestering activities than does density gradient centrifugation. The results from the gradient separations indicate that microsomes in the upper layers were more active in both Ca\textsuperscript{++}-accumulation and ATP-splitting than were microsomes in the lower layers. There was a very small, but definite improvement in ATPase and Ca\textsuperscript{++}-sequestering activities caused by density gradient purification of 0.6 M KCl-extracted crude microsomes. The activities exhibited by this density gradient fraction were the highest encountered in this entire study. However, the improvement in activity caused by density gradient centrifugation of KCl-extracted crude microsomes is small, and it is problematical whether this small increase in specific activity is always worth the time and loss in yield experienced during a subsequent density gradient purification. Inclusion of 0.15 M KCl in the gradient did not have any detectable effect on the ATPase and Ca\textsuperscript{++}-accumulating activity of the density gradient-separated layers. The 0.15 KCl was included in the sucrose density gradients to test whether inclusion of KCl might solubilize some of myosin evidently contaminating crude muscle microsomal preparations. Such solubilization should cause better separation between microsomes and myosin on the density gradients, but the results indicate that this did not occur. This study on purification of muscle microsomes suggests that direct extraction of crude microsomes with 0.6 M KCl will produce more highly purified microsomes than most preparations described in the literature. If the highest possible degree of purity is desired even at the sacrifice of some yield, the KCl-extracted
microsomes may be slightly further purified by density-gradient centrifugation on sucrose gradients.

Post-Mortem Studies

After the studies just described had permitted selection of a satisfactory method for isolation and purification of muscle microsomes, a second investigation was conducted to characterize the post-mortem behavior of skeletal muscle microsomes. In view of the results described in the preceding section, all microsomal preparations studied in this investigation were prepared by 0.6 M KCl extraction of crude microsomes without any prior or subsequent density gradient purification. Microsomal preparations from three species were utilized. Ca\(^{++}\)-accumulation and ATPase activity of microsomal fractions isolated by differential centrifugation from rabbit back and leg muscle or porcine longissimus dorsi are presented in Tables 8 and 9, respectively. Inspection of Tables 8 and 9 reveals that microsomes isolated from muscle that had been stored 24 hours at either 2\(^\circ\) or 23\(^\circ\)C have less than 25% of the Ca\(^{++}\)-sequestering ability possessed by microsomes isolated immediately after death. However, little or no difference exists between ATPase activities of microsomal fractions isolated from muscle sampled immediately after death and those isolated 24 hr following exsanguination. Thus, sarco-tubular vesicles prepared from post-mortem muscle constitutes another example of uncoupling of the ATPase activity and the Ca\(^{++}\)-accumulating ability of muscle microsomes. Electron micrographs of both rabbit and porcine microsomal fractions prepared immediately after death and after 24 hr of post-mortem storage at either 2\(^\circ\) or 23\(^\circ\)C are presented in
Table 8. Post-mortem changes in Ca$^{++}$-sequestration and ATPase activities of microsomes isolated from rabbit skeletal muscle stored at different temperatures

<table>
<thead>
<tr>
<th>Time and temperature of post-mortem storage</th>
<th>ATPase activity $\mu$moles P$_i$/mg protein/min [Mean (8) ± S.E.]</th>
<th>Ca$^{++}$ sequestration $\mu$moles Ca$^{++}$/mg protein [Mean (8) ± S.E.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>.652 ± .010</td>
<td>2.30 ± 0.05</td>
</tr>
<tr>
<td>24 hr, 2°C</td>
<td>.644 ± .016</td>
<td>0.57 ± 0.06</td>
</tr>
<tr>
<td>24 hr, 23°C</td>
<td>.636 ± .010</td>
<td>0.33 ± 0.04</td>
</tr>
</tbody>
</table>

*Conditions of assay: 0.1 M KCl, 30 mM Hepes (pH 7.2), 0.05 mg protein/ml, 5 mM ATP, 5 mM MgCl$_2$, 5 mM K$_2$C$_2$O$_4$, 0.15 mM $^{45}$CaCl$_2$; total volume, 5 ml; 25°C. Assays were run for 15 min.

Table 9. Post-mortem changes in Ca$^{++}$-sequestration and ATPase activities of microsomes isolated from porcine skeletal muscle stored at different temperatures

<table>
<thead>
<tr>
<th>Time and temperature of post-mortem storage</th>
<th>ATPase activity $\mu$moles P$_i$/mg protein/min [Mean (8) ± S.E.]</th>
<th>Ca$^{++}$ sequestration $\mu$moles Ca$^{++}$/mg protein [Mean (8) ± S.E.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>.637 ± .004</td>
<td>2.45 ± 0.06</td>
</tr>
<tr>
<td>24 hr, 2°C</td>
<td>.592 ± .010</td>
<td>0.86 ± 0.12</td>
</tr>
<tr>
<td>24 hr, 23°C</td>
<td>.575 ± .007</td>
<td>0.69 ± 0.08</td>
</tr>
</tbody>
</table>

*Conditions of assay: 0.1 M KCl, 30 mM Hepes (pH 7.2), 0.05 mg protein/ml, 5 mM ATP, 5 mM MgCl$_2$, 5 mM K$_2$C$_2$O$_4$, 0.15 mM $^{45}$CaCl$_2$; total volume, 5 ml; 25°C. Assays were run for 15 min.

Figures 8-13. In all cases, the microsomal fraction consisted of vesicular elements of various sizes. It was generally observed that microsomes prepared from post-mortem muscle contained a lower proportion of flattened vesicle than microsomes prepared immediately after death. However, on
Figure 8. Microsomes (8,200 - 53,679 x g fraction, extracted twice with 0.6 M KCl) isolated from rabbit skeletal muscle immediately after death. Samples were fixed with glutaraldehyde and postfixed with osmium tetroxide. Section was stained with methanolic uranyl acetate. Magnification: X 29,000.

Figure 9. Microsomes (8,200 - 53,679 x g fraction, extracted twice with 0.6 M KCl) isolated from rabbit skeletal muscle after storage for 24 hr post-mortem at 2°C. Samples were fixed with glutaraldehyde and postfixed with osmium tetroxide. Section was stained with methanolic uranyl acetate. Magnification: X 43,700.

Figure 10. Microsomes (8,200 - 53,679 x g fraction, extracted twice with 0.6 M KCl) isolated from rabbit skeletal muscle after storage for 24 hr post-mortem at 23°C. Samples were fixed with glutaraldehyde and postfixed with osmium tetroxide. Section was stained with methanolic uranyl acetate. Magnification: X 79,800.
the basis of evidence available thus far, the loss of Ca\textsuperscript{++}-accumulating ability in post-mortem muscle microsomes cannot be related to any structural change. These results are in agreement with the findings of Greaser et al. (1967) who reported that the Ca\textsuperscript{++}-sequestering ability of microsomal fractions isolated from porcine skeletal muscle decreased with increasing post-mortem storage, the largest decrease occurring after 2 hr post-mortem.

Very little is known about the effect of chemical fixation on ultrastructure of biological membranes, and it is conceivable that fixation of vesicular preparations for electron microscopy destroys some of the structural differences which exist between at-death and 24-hour microsomes. Lenard and Singer (1968) have shown that several fixatives (glutaraldehyde; osmium tetroxide; glutaraldehyde followed by osmium tetroxide, and potassium permanganate) produce significant and parallel conformational changes in red blood cell membranes. However, in this study all samples for electron microscopy were prepared in the same way so structural differences observed should be ascribed to post-mortem storage of the muscle.

A limited study was conducted on the ATPase and Ca\textsuperscript{++}-accumulating activities of microsomal preparations prepared from rabbit psoas or beef semitendinosus muscle immediately after death, at the onset of post-mortem tension development, and after maximum post-mortem tension development. Examination of the histograms presented in Figures 14 and 15 show that skeletal muscle microsomes isolated from muscle strips that had just begun to develop post-mortem tension have lost most of their ability to
Figure 14. Ca$^{++}$ sequestration and ATPase activities of microsomes isolated from rabbit psoas muscle immediately after death, at the onset, and after the development of maximum tension during rigor. Temperature = 37°C.
ATPASE ACTIVITY AND Ca++ SEQUESTRATION IN MICROSOMES ISOLATED FROM RABBIT PSOAS MUSCLE—BEFORE, AT THE START AND AFTER THE DEVELOPMENT OF TENSION AT 37°C
Figure 15. Ca\textsuperscript{++} sequestration and ATPase activity of microsomes isolated from beef \textit{semitendinosus} muscle immediately after death, at the onset, and after the development of maximum tension during rigor.
KEY: ATPASE
Ca++ SEQUESTRATION

ATPASE ACTIVITY AND Ca++ SEQUESTRATION IN MICROSOMES ISOLATED FROM BEEF SEMITENDINOSUS MUSCLE BEFORE, AT THE ONSET, AND AFTER THE DEVELOPMENT OF TENSION
accumulate calcium but have suffered no loss in ATPase activity. This loss of Ca\(^{++}\)-sequestering ability occurred at post-mortem storage temperatures of both 2\(^{\circ}\) and 37\(^{\circ}\)C in bovine muscle and at the 37\(^{\circ}\)C storage temperature in rabbit muscle. However, the time of the onset of tension development varied widely from 2.4 hr post-mortem in rabbit muscle at 37\(^{\circ}\)C to 6.9 hr post-mortem in bovine muscle at 2\(^{\circ}\)C. This suggests that a correlation exists between onset of post-mortem tension development and loss of the ability of a muscle's sarcoplasmic reticulum to accumulate Ca\(^{++}\). The results of this experiment, therefore, parallel the earlier results obtained on post-mortem rabbit and porcine microsomes since ATPase activities of the microsomes remained high even though Ca\(^{++}\)-sequestering ability was lost.
DISCUSSION

Isolation and Purification of Skeletal Muscle Microsomes

The results of this study confirm previous reports that a microsomal fraction which possesses a very active Mg$^{++}$-stimulated ATPase activity and the ability to accumulate Ca$^{++}$ can be sedimented from a muscle homogenate between 8,200 x g for 20 min and 53,679 x g for 120 min. The Ca$^{++}$-sequestering and Mg$^{++}$-modified ATPase activities of the microsomal preparations in this study are comparable with values obtained for similar preparations by other workers (Carsten and Mommaerts, 1964; Ebashi and Yamanouchi, 1964; Greaser et al., 1967; Harigaya et al., 1968; Hasselbach and Seraydarian, 1966; Martonosi, 1968b; Martonosi et al., 1968; Martonosi and Feretos, 1964a,b; Seraydarian and Mommaerts, 1965; and Sréter and Gergely, 1964). These previous studies have shown that muscle microsomal preparations are capable of accumulating 1.5 to 3.0 μmoles of Ca$^{++}$ per mg of microsomal protein in oxalate-stimulated systems and have Mg$^{++}$-modified ATPase activities of 0.6 μmoles P$_i$ liberated per mg microsomal protein per min. Thus, even the relatively crude microsomal fractions isolated by differential centrifugation possess substantial Ca$^{++}$-accumulating activity, particularly when considered in view of Hasselbach's (1964b) estimation that only 0.1 μmoles of Ca$^{++}$/cm$^3$ of muscle needs to be transported per 300 msec at 20°C to cause cessation of contraction. If there is 4 mg of reticular protein per cm$^3$ of muscle (Weber et al., 1964), this suggests that a Ca$^{++}$-accumulating ability of only 0.025 μmoles/mg of reticular protein/300 msec is sufficient for relaxation.
Electron microscopic examination of the crude microsomal fraction prepared in this study has demonstrated that this fraction is heterogeneous. Although crude microsomes consisted mainly of vesicular material of different shapes and sizes, similar in appearance to preparations made from rabbit or rat muscle by other workers (Ebashi and Lipmann, 1962; Engel and Tice, 1966; Martonosi, 1964; Nagai et al., 1960), closer examination of the bottom of the microsomal pellet revealed the presence of small numbers of mitochondria and mitochondrial fragments. Furthermore, the results of the KCl-extraction experiments in this study showed that crude microsomal preparations may contain as much as 20 to 30% of their protein as myosin. After KCl-extraction, the Ca$^{++}$-accumulating abilities of the microsomes increased by 35-75% and their Mg$^{++}$-modified ATPase activities increased by 60-90%. Electron microscopic examinations of the KCl-extracted microsomes indicated that KCl-extracted microsomes appeared to contain less electron dense material, and in some cases had a higher proportion of flattened vesicles than crude microsomes. The exact significance of the electron dense material in microsomes is not clear although Seraydarian and Mommaerts (1965) have suggested that such microsomes may be filled with Ca$^{++}$ and thus unable to accumulate additional Ca$^{++}$ in in vitro assays. If so, it is not clear why KCl extraction should cause disappearance of such vesicles from microsomal preparation. The finding of myosin-like protein in crude muscle microsomal preparations made by homogenization in sucrose is in contrast to the results of Uchida et al. (1965) who suggest the use of sucrose media to prevent the occurrence of myosin in crude microsomal preparations. The reason for this discrepancy
is not clear, but in retrospect, it seems possible that differences in time of initial homogenization of the minced muscle may account for this. Seventy sec of homogenization time were used in this study whereas Uchida et al. (1965) used only 30 sec. It is probable that longer homogenization causes more extensive fragmentation of myosin filaments and thereby results in some myosin not being sedimented at centrifugal forces below 8,200 x g, but then being centrifuged down at higher centrifugal forces where the microsomes are also sedimented. Homogenization time was not one of the factors studied in this investigation, and indeed, it appears that very few systematic studies have been done on the influence of homogenization time on the composition of subcellular fractions prepared from muscle homogenate by differential centrifugation.

Density gradient centrifugation of either the crude microsomal fraction or the KCl-extracted microsomal preparation through a continuous (20 - 60%) sucrose gradient resolved the microsomal fraction into two subfractions. Both subfractions possessed Ca\(^{++}\)-sequestering ability and ATPase activity, but the upper layer or less dense fraction was always the more active of the two. In fact, the upper layer obtained from KCl-extracted sucrose gradient-purified microsomes was the most active fraction prepared in this entire study, both in terms of Ca\(^{++}\)-sequestration and in Mg\(^{++}\)-modified ATPase activity. The upper layer obtained from sucrose density gradient centrifugation had 30-60% more Ca\(^{++}\)-sequestering ability and 20-35% more Mg\(^{++}\)-modified ATPase activity than did the microsomal preparation before application to the sucrose gradient. This was true for the crude microsomal preparation but the KCl-extracted preparation was not purified to the same extent by density gradient centrifugation. The
increase in Ca^{++}-accumulating ability or ATPase activity caused by density gradient purification in this study was less than that effected by sucrose density gradient purification in the study of Yu et al. (1968). In fact, the results of the present study suggest that KCl-extraction causes greater purification than density gradient centrifugation. It seems probable that, among other things, density-gradient purification effectively removes mitochondrial contamination from microsomal preparations. This conclusion is substantiated by the fact that in this study neither mitochondria nor mitochondrial fragments were ever observed in electron micrographs of the upper layer obtained from sucrose density gradient centrifugation. Consequently, the relatively small increase in specific activity caused by density gradient purification of muscle microsomes in the present study compared to that achieved in Yu's study may be due to the fact that the particular combination of species, homogenization time, and differential centrifugation conditions in this study resulted in a microsomal fraction that contained relatively more myosin and relatively less mitochondrial contamination than did the similar combination of conditions in Yu's study.

From the considerations discussed above it appears that the best method for preparation of reasonably pure sarcotubular membranes from either rabbit or porcine muscle would be as follows: 1) Homogenization of minced muscle in 0.25 M sucrose, 0.05 M Tris-HCl, 1 mM EDTA, pH 7.6, for a total of 70 sec. 2) Centrifugation at 10,000 x g for 30 min to remove heavy cytological and mitochondrial debris. 3) Centrifugation at approximately 60,000 x g for 2 hr to sediment the crude microsomal fraction. 4) Extraction of the crude microsomal pellet with 0.6 M KCl followed by centrifugation at 100,000 x g for 60 min to pellet the
extracted microsomes. 5) Repetition of step 4. 6) Layering of the KCl-extracted microsomal fraction onto a continuous (20 - 60%) sucrose density gradient, followed by centrifugation at 106,000 x g for 120 min. This density gradient purification could also be accomplished on a larger scale by use of zonal ultracentrifugation.

The evidence obtained in this study still does not permit a decision concerning the absolute "purity" of microsomes prepared according to the preceding method. "Purity", at least for muscle microsomes, will probably ultimately be defined in terms of percentage of membranous elements actively sequestering Ca++. Therefore, in order to more accurately determine the purity of sarcotubular preparations made according to preceding criteria, it will probably be necessary to incubate the fractions with Ca++ and then determine the percentage of vesicles which have actively sequestered Ca++. One possible method for doing this would be to incubate the vesicles with Ca++ in the presence of oxalate until Ca++ accumulation has ceased and then to examine the preparation in the electron microscope to determine what percentage of the vesicles contains a Ca++-oxalate precipitate. Once the purity of the preparation has been established, it will be interesting to determine the protein and phospholipid composition of Ca++-accumulating membranes. Evidence on the mode of action whereby the sarcotubular membranes accumulate Ca++ will also probably have to await the separation and demonstration of purified Ca++-accumulating membrane preparations.
Post-Mortem Studies

The results of the study on post-mortem muscle confirm and extend the work of Greaser et al. (1967) who found that porcine skeletal muscle microsomes lost 90% of their ability of accumulate Ca\(^{++}\) within 24 hr after death. The results of the present study demonstrate that microsomes isolated from both rabbit and porcine muscle after 24 hr post-mortem have lost their ability to accumulate Ca\(^{++}\). Furthermore, it has been shown that although microsomes isolated from post-mortem muscle have lost the ability to accumulate Ca\(^{++}\), their Mg\(^{++}\)-modified ATPase activity remains high and even appears to increase slightly with post-mortem time. Thus, post-mortem muscle is another example of the uncoupling of the Mg\(^{++}\)-modified ATPase activity of muscle microsomes from the Ca\(^{++}\)-accumulating ability of these microsomes. Moreover, the results of the present study suggest that the process of tension development during rigor may be closely correlated to inactivation of the sarcotubular fraction; this inactivation causes release of Ca\(^{++}\) and initiation of a contractile-like response. Thus, it was shown that Ca\(^{++}\)-accumulating ability of microsomes isolated from post-mortem porcine muscle strips was lost just prior to the time that these strips began to develop post-mortem tension.

In view of the known lability of the Ca\(^{++}\)-accumulating ability of muscle microsomes to storage of any kind, it is not surprising that the Ca\(^{++}\)-accumulating ability of microsomes isolated from post-mortem is low. However, it is surprising that the loss of the Ca\(^{++}\)-sequestering ability occurs so rapidly, with 80% of the Ca\(^{++}\)-sequestering ability of rabbit microsomes being lost within 3 hr after death. Many of the chemical
changes which occur in muscle during the first 24 hr post-mortem have been
summarized (Briskey, 1959; Goil, 1968). It has been shown that pH in
muscle decreases rapidly after death from 7.0 to values as low as 5.0
within 24 hr post-mortem (Bendall, 1963; Briskey, 1959; Lawrie, 1960).
Furthermore, it has been reported that the Ca\(^{++}\)-accumulating ability of
sarcotubular vesicles is lost after exposure to pH values below 4.5 or
above 9.0 (Ebashi, 1961; Hasselbach, 1964b; Marsh, 1952). Therefore, it
seems probable that the low pH which exists in post-mortem muscle causes
some kind of disorganization or disruption of the particular phospholipid-
protein complex that is responsible for the Ca\(^{++}\)-sequestering ability of
the sarcotubular membranes (Martonosi, 1968a; Martonosi et al., 1968).
Loss of the proper three-dimensional structure of the sarcotubular
membrane could then result in loss of Ca\(^{++}\)-accumulation by the membranes.
Post-mortem destruction of the sarcotubular membranes might also be aided
or accelerated by hydrolytic enzymes (lipases, phospholipases, proteases)
released after death from lysosomes (Neelin and Ecobichon, 1966).

If such post-mortem alteration in the membrane structure of the
sarcotubular vesicles does occur, it is difficult to detect by electron
microscopy. Although there tended to be a lower proportion of flattened
vesicles in the post-mortem microsomes, there were no other marked
differences in structure between vesicles isolated from muscle immediately
after death and those isolated 24 hr post-mortem. Greaser et al. (1967)
previously reported that the structure of vesicles isolated from porcine
muscle 24 hr post-mortem was similar to that of vesicles isolated
immediately after death. Therefore, whatever the alteration that causes
loss of Ca\(^{++}\)-sequestering ability in post-mortem muscle microsomes, this
alteration is either not preserved by the fixation process, or it is below
the limits of resolution obtainable with existing sample preparation
techniques.
The preparation of skeletal muscle microsomes was investigated in an effort to determine those conditions that would produce the most active and homogeneous preparations. In the case of muscle microsomes, purity is defined in terms of Ca$^{++}$-sequestering ability and Mg$^{++}$-modified ATPase activity, with the purer preparation exhibiting a greater ability to bind Ca$^{++}$ and a higher Mg$^{++}$-modified ATPase activity. Both rabbit and porcine muscle was used. A crude microsomal fraction isolated between 8,200 x g for 30 min and 53,678 x g for 2 hr had an active Ca$^{++}$-accumulating ability and a very active Mg$^{++}$-modified ATPase activity. However, examination by electron microscopy showed that this crude microsomal fraction was heterogeneous. The upper part of the pellet consisted of vesicular material of various shapes and sizes, but the bottom of the pellet contained some mitochondria and mitochondrial fragments. Layering of a resuspended crude microsomal pellet onto a continuous (20 - 60%) sucrose density gradient followed by centrifugation at 106,900 x g for 90 min resulted in the formation of two zones. Both zones possessed Ca$^{++}$-accumulating ability and ATPase activity, but the upper was more active than the lower. When compared to the crude microsomal fraction, the upper zone had 30-60% higher Ca$^{++}$-accumulating ability and 20-35% higher Mg$^{++}$-modified ATPase activity. Electron microscopy of the upper zone indicated that it did not contain any mitochondria or mitochondrial fragments. Subjecting the upper zone to centrifugation through a second continuous (20 - 60%) sucrose density gradient resulted in only slight improvement in either Ca$^{++}$-accumulating ability or Mg$^{++}$-modified ATPase activity.
It was discovered, however, that 0.6 M KCl extraction of the microsomes from the upper density gradient zone caused a 15% increase in Ca\(^{++}\)-sequestering ability and 40% increase in Mg\(^{++}\)-modified ATPase activity. The protein extracted by this KCl treatment resembled myosin, in that it had an ATPase activity which was inhibited by Mg\(^{++}\) and activated by Ca\(^{++}\). Moreover, the protein sedimented in the analytical ultracentrifuge with the sedimentation coefficient of 6 S, similar to the sedimentation coefficient of myosin. Extraction of crude microsomes, before density gradient purification, with 0.6 M KCl also caused 35-75% increase in Ca\(^{++}\)-accumulating ability and a 60-90% increase in Mg\(^{++}\)-modified ATPase activity. Density gradient centrifugation of the KCl-extracted microsomes through a continuous (20 → 60%) sucrose gradient resulted in an additional 7% increase in Ca\(^{++}\)-sequestering ability and a 5% increase in Mg\(^{++}\)-modified ATPase activity. Density gradient purification of the KCl-extracted microsomes produced microsomes having the highest Ca\(^{++}\)-accumulating ability and ATPase activity observed in this study. A uniform concentration of 0.15 M KCl was included in the continuous (20 → 60%) sucrose gradient to determine whether this would solubilize myosin and effect purification of microsomes in a single density gradient centrifugation without any KCl-extraction. The inclusion of 0.15 M KCl in the sucrose gradient had no detectable effect on density gradient purification of either crude microsomes or KCl-extracted microsomes.

The results of this study suggest that the following procedure is best for the preparation of pure skeletal muscle microsomal fractions: 1) homogenization of muscle in 0.25 M sucrose, 0.05 M Tris, 1 mM EDTA, pH 7.6 for a total of 70 sec; 2) centrifugation of the homogenate at 10,000 x
for 30 min to remove heavy cytological and mitochondrial debris; 3) centrifugation at approximately 50,000 x g for 120 min to sediment the crude microsomal fraction; 4) extraction of the crude microsomal pellet with 0.6 M KCl, followed by pelleting at 100,000 x g for 1 hr; 5) repetition of step 4; 6) layering the KCl-extracted pellet, suspended in 0.25 M sucrose, onto a continuous (20 - 60%) density gradient followed by centrifugation at 106,900 x g for 120 min.

Changes in the Ca\textsuperscript{2+}-accumulating abilities and Mg\textsuperscript{2+}-modified ATPase activities of microsomal fractions prepared from rabbit, porcine, or bovine muscle after varying times of post-mortem storage up to 24 hr were also studied. Ca\textsuperscript{2+}-accumulating ability of muscle microsomes from all three species decreased rapidly with increasing post-mortem time, and after 24 hr post-mortem, was only 25% of the initial Ca\textsuperscript{2+}-accumulating ability. The loss of Ca\textsuperscript{2+}-accumulating ability began as early as 3 hr after death in rabbit muscle microsomes. The Mg\textsuperscript{2+}-modified ATPase activity of muscle microsomes, on the other hand, remained high during post-mortem storage and even appeared to increase slightly with increasing post-mortem time up to 24 hr. Since loss of Ca\textsuperscript{2+}-accumulating ability occurred just prior to development of post-mortem tension, it seems probable that release of Ca\textsuperscript{2+} from the sarcoplasmic reticular membranes initiates post-mortem tension development.
CONCLUSIONS

As a result of this study, the following conclusions seem warranted:

1. The crude skeletal muscle microsomal fraction prepared by homogenization in sucrose media and differential sedimentation between 8,200 x g for 30 min and 53,678 x g for 2 hr contains mitochondrial contamination, as ascertained by electron microscopy. This contamination is very small since the Mg$^{++}$-stimulated ATPase activity of the crude microsomal fraction is not inhibited by sodium azide.

2. Layering of the resuspended crude microsomal pellet onto a continuous (20 → 60%) sucrose density gradient followed by centrifugation at 106,900 x g for 90 min results in the formation of two zones. Both zones possess Ca$^{++}$-accumulating ability and Mg$^{++}$-modified ATPase activity. The upper or less dense zone contains slightly more than half of the total protein applied to the sucrose density gradient and also exhibits slightly greater Ca$^{++}$-accumulating ability and ATPase activity than the lower or denser zone. Application of microsomes obtained from the upper zone of the first sucrose gradient onto a second continuous (20 → 60%) sucrose density gradient results in only an 8% increase in the Ca$^{++}$-accumulating ability and a 13% increase in the Mg$^{++}$-modified ATPase activity of the microsomes. Hence a single density gradient purification step, using a continuous (20 → 60%) sucrose gradient, produces almost all the purification of crude muscle microsomes that it is possible to obtain by
density gradient centrifugation.

3. Extraction of microsomes from the upper density gradient zone with 0.6 M KCl results in a 30-60% increase in Ca\(^{++}\)-accumulating ability and a 20-25% increase in Mg\(^{++}\)-modified ATPase activity. The protein extracted from the microsomes by 0.6 M KCl resembles myosin in that it has an ATPase activity which, at 50 mM KCl, is inhibited by Mg\(^{++}\), but activated by Ca\(^{++}\). The protein also exhibits a sedimentation coefficient of 6 S, very close to the sedimentation coefficient of myosin. Extraction of crude microsomes with 0.6 M KCl results in a 35-75% increase in Ca\(^{++}\)-sequestering ability and a 60-90% increase in Mg\(^{++}\)-modified ATPase activity. Centrifugation of the KCl-extracted microsomes through a continuous (20-60%) sucrose gradient results in a microsomal preparation which had the highest Ca\(^{++}\)-accumulating ability and ATPase activity observed in this study.

4. In view of the preceding conclusions, the best method for preparation of "pure" microsomes is: 1) homogenization of minced muscle in 0.25 M sucrose, 0.05 M Tris, 1 mM EDTA for 70 sec, 2) centrifugation of the homogenate at 10,000 \(\times g\) for 30 min, 3) centrifugation of the 10,000 \(\times g\) supernatant for 120 min at approximately 60,000 \(\times g\) to sediment a crude microsomal fraction, 4) extraction of the crude microsomal fraction with 0.6 M KCl followed by centrifugation at 100,000 \(\times g\) to pellet the extracted microsomes, 5) repetition of step 4. A slight additional purification can be achieved by centrifugation of the microsomes
from step 5 through a continuous (20 → 60%) sucrose density gradient.

5. Microsomes isolated from porcine or rabbit skeletal muscle after 24 hr of post-mortem storage exhibit only 25% of the Ca$^{++}$-sequestering ability possessed by microsomal fractions prepared from at-death muscle. Rabbit and beef skeletal muscle microsomes begin to lose their Ca$^{++}$-sequestering ability just prior to the onset of post-mortem tension. This suggests that loss of the ability of the sarcoplasmic reticulum in post-morten muscle to accumulate Ca$^{++}$ is responsible for initiating post-mortem tension development.
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